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14. ABSTRACT

Malaria is the primary infectious disease threat facing the U.S. soldier, and is the leading cause of all causalities during tropical deployments. The long-term objective of this project is to identify and prepare the malaria parasite forms causing severe anemia, and then apply functional genomics and bioformatics tools to identify 15 to 30 proteins that could form the basis for an effective vaccine at both the pre-erythrocytic and blood stages of malaria infection. The project will then evaluate these lead candidates for their recognition by sera collected from immune individuals, in order to identify the leading 3 to 5 candidates for a blood stage vaccine that prevents severe malaria anemia.

15. SUBJECT TERMS
Severe malaria, *P. falciparum,* microarrays, proteomic, vaccines

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Table of Contents

Page

INTRODUCTION: Malaria is the primary infectious disease threat facing the U.S. solider, and is the leading cause of all casualties during tropical deployments. The long-term objective of this project was to identify and prepare the malaria parasite forms causing severe anemia, and then apply functional genomics and bioinformatics tools to identify 15 to 30 proteins that could form the basis for an effective vaccine at both the pre-erythrocytic and blood stages of malaria infection. The project was to then evaluate these lead candidates for their recognition by sera collected from immune individuals, in order to identify the leading 3 to 5 candidates for a blood stage vaccine that prevents severe malarial anemia.

This project has achieved an advanced stage of candidate antigen identification for a vaccine against malarial anemia. During the course of the project, we successfully applied functional genomics and bioinformatic tools to identify ~50 genes and proteins that were upregulated in parasites causing malaria anemia, and used additional PCR-based tools to verify that anemia parasites express a subset of these at higher levels. We currently have the tools in place to complete the down-selection of these candidate genes/proteins in the near future to determine which of these has optimal features as a potential vaccine candidate. The project has also allowed us to pioneer new technologies for the study of malaria parasites infecting humans, such as high throughput sequencing, that have poised us to accelerate studies of the parasites causing severe disease and the effort to develop interventions including vaccines to prevent malaria disease and death.

BODY:

Regulatory Status

Our DOD protocol titled, "Antigens for a Vaccine that Prevents Severe Malaria" describes the functional genomics and immunoreactivity studies that we are performing on malaria parasites. After extensive revisions during the first grant year, it now supports laboratory work being conducted only at the Seattle Biomedical Research Institute in the U.S., and includes sample processing, immunoparasitology, and functional genomics studies on samples collected under a separately IRB-approved longitudinal cohort study. The longitudinal cohort study has previously been supported by funds from the National Institutes of Health and the Bill & Melinda Gates Foundation and is currently supported by a Grand Challenges grant from the Foundation for the National Institutes of Health.

We submitted the DOD protocol for continuing review to our local IRB (Western IRB in Olympia, WA) in November 2008 and received continuing approval on December 18, 2008 (annual approval expires January 6, 2010). We will plan to keep the protocol open for ongoing analysis, as well as, we intend to submit a renewal proposal to DOD for this project.

Microarray analysis of field samples

We processed 49 field samples after extracting RNA from peripheral blood from children in the Muheza cohort by in-house spotted microarrays, using a commercially available set of more than 8000 70-mers (Bozdech, et al, 2003), with the addition of 1,920 additional probes designed to capture the diversity of PfEMP1 genes, field isolates, and human genes. We applied a reference-based experimental design in which each sample was compared to the same pool of field and lab-adapted isolates in a two-channel setup, enabling the indirect comparison of transcription levels among different samples. Most samples were analyzed by multiple slides, and in total 159 slides from 46 patients were processed. After the

implementation of strict quality control criteria, which resulted in the exclusion of lower quality slides, our final dataset included 69 slides from 31 patients (Table IA: Clinical Data for Muheza Samples). A limitation of this analysis was the absence of severe anemia cases in the final dataset, although several samples were classified as moderate anemia. In addition, we observed that the concordance between multiple probes of same gene was not always good.

Table 1A: Muheza Samples	Anemic Group (n=6)	Non-anemic Group (n=25)
HGB (g/dL)	6.88 +/- 0.55	12.67+/-1.95
BS (parasites/200WBC)	2400 +/- 1404	4377 +/- 3660
Age (weeks)	71.5 +/- 34.1	106 +/- 39.9

Therefore, we performed a pilot experiment where 4 of these samples were analyzed by genomic tiling arrays printed by Nimblegen. The custom design was based on the 5.2 version of the 3D7 reference strain genome, and included 385,000 60-mer probes covering predicted exons, introns and intragenic regions. This experiment yielded encouraging results, including a good correlation in the levels of gene expression with the spotted array data for those same 4 samples. Based on these data, we analyzed the gene expression of 10 anemic and 10 non-anemic children samples from the Morogoro cohort using genomic tiling arrays (Table 1B: Clinical Information of Morogoro Samples).

Table 1B	Anemic Group (n=10)	Non-anemic Group (n=10)
HGB (g/dL)	5.2 +/- 1.04	10.83 +/- 2.84
BS (parasites/200WBC)	9590 +/- 9186	3088 +/- 2860
Age (weeks)	156 +/- 208	89 +/- 52

The anemic samples were selected to represent the most extreme cases of low hemoglobin available (excluding those with complicating factors such as coinfection). The resulting data displays high concordance in the signal between the multiple probes from individual genes and was deemed to be of excellent quality.

Multiple bioinformatic tools (including Acuity, GenePattern, TM4, and R language code developed in site) were applied to both datasets in order to identify genes with apparent up-regulation in parasites isolated from anemic episodes as compared to parasites isolated from children with normal hemoglobin levels (>8 g/dL). The initial gene lists were parsed on the basis of their known or predicted function, presence of signal peptides or transmembrane domains, or evidence of export to the host cell. A total of 26 genes were selected to be further analyzed by qPCR in a larger number of samples, and these analyses are currently being completed. Genes whose increased expression in anemic vs. non-anemic samples was confirmed by qPCR are being further analyzed by multiple approaches, as shown in Table 2.

PlasmoDB Gene ID	Description	qPCR	Cloning	Protein Expression	Bioplex (Patient Sera Reactivity)	Mouse Antibody	Flow Cytometry	IFA
PFD1120c	Etramp family	√	√	√	√	√	√	√
PFE1590w		√						
MAL7P1.174	pHIST/RESA family	√	√	√	√	√	√	
PFF0075C		√						
PFD1170C		√						
MAL13P1.58		√						
PFD1180W		√						
PFE1605W		√						
PF11_0037		√						
PFB0095C	PfEMP3	√	√	√	√	√		
PF10_0155	Enolase	√	√	√	√			
PF10_0160	FIKK kinase	√	√					
PF13_0010	Glycophorin binding protein family	√						
PF14_0010		√						
PF10_0159		√						
PFB1045W	Truncated var	√						
MAL7P1.186		√						
PF11_0362	Protein phosphatase 2	√						
PF14_0528	Hemolysin	√						
PFB0115W	Conserved hypothetical	√						
PF14_0650	Conserved hypothetical	√						
PF14_0544	Conserved hypothetical	√						
PF11_0508	Exported hypothetical	√						
PF14_0731	Hypothetical	√						
MAL7P1.173	Hypothetical	√						
MAL13P1.202	hypothetical	√						

Table 2: Status of vaccine candidates

Of note is PFD1120c/ETRAMP4, which was identified as being up-regulated in anemic samples from both the Muheza and Morogoro cohorts by microarray analyses, and confirmed by qPCR (Figure 1A and B).

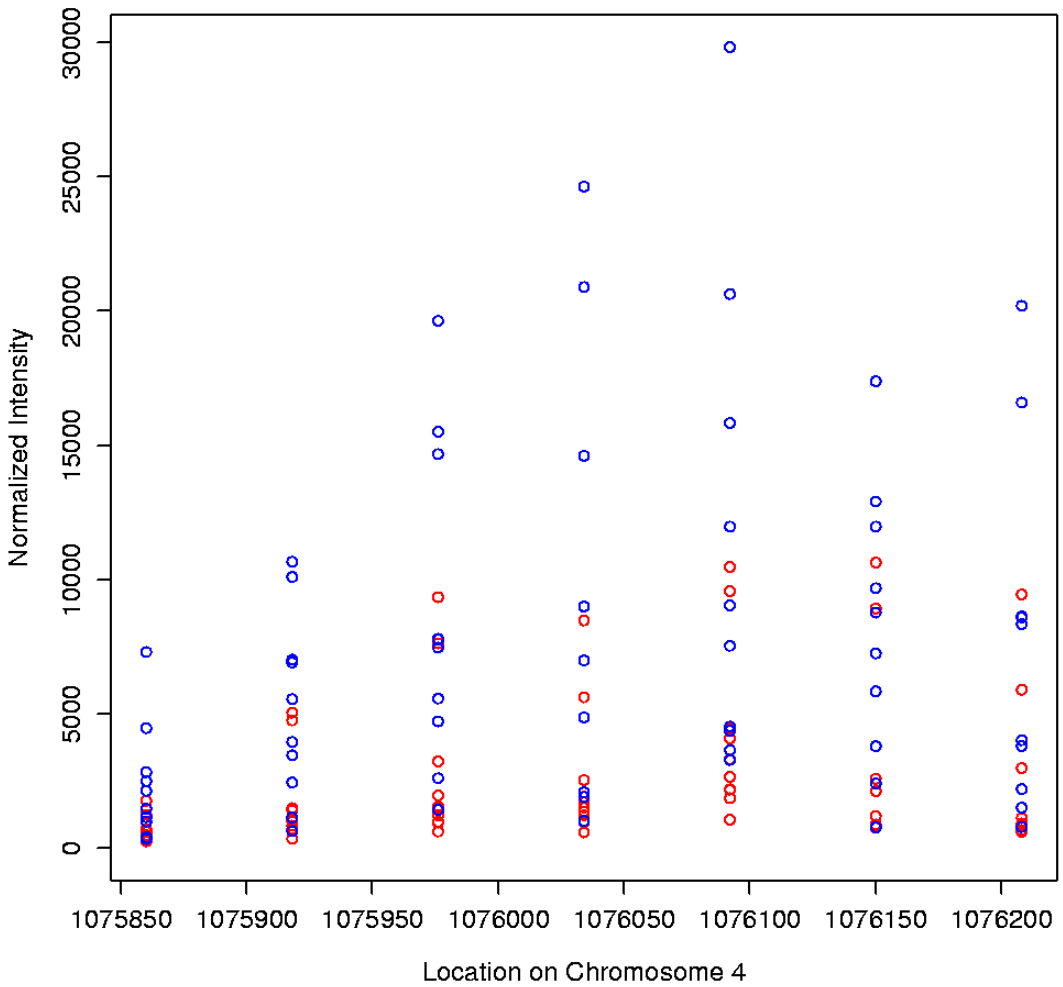

Figure 1A: Probe intensities for PFD1120c in Morogoro tiling arrays. Blue = anemic samples, red = non-anemic sample

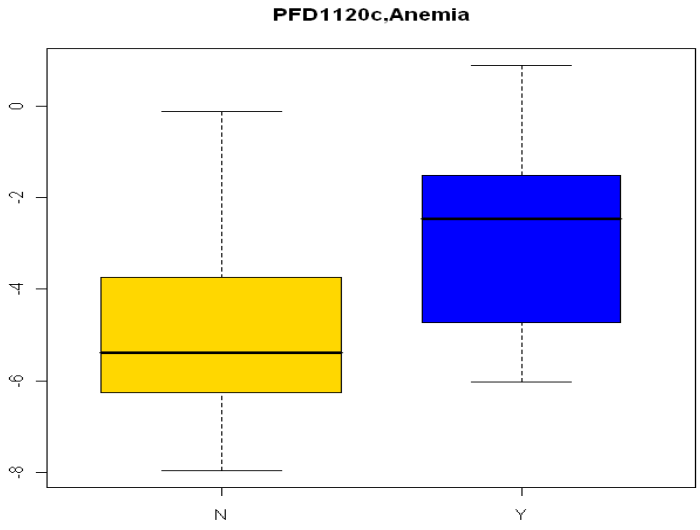

Figure 1B: qPCR Validation of Microarray Results

PFD1120c/ETRAMP4 is a highly expressed, immunogenic membrane protein that, in lab-adapted strains, has been reported to be localized to the parasitophorous vacuole membrane (PVM), at the interface of the parasite and the host cytoplasm. The protein was also observed by immunofluorescence to cytoplasmic vesicles or 'blebs' extending from the PVM, which the authors hypothesized could correspond to export vesicles (Spielmann et al. 2003, Birago et al 2003). To investigate the potential surface localization of this protein, we cloned and expressed a full-length construct by a commercially available *in vitro* transcription and translation system. This protein was used to raise mouse antibodies, and we are currently attempting to determine its potential surface localization in erythrocytes infected with parasites isolated from an anemic child by flow-cytometry and immunofluorescence assays. In addition, this protein was included in our Bioplex serological assays, which allowed us to verify that antibodies against this protein exist in individuals that have a history of malaria infections (Figure 1C).

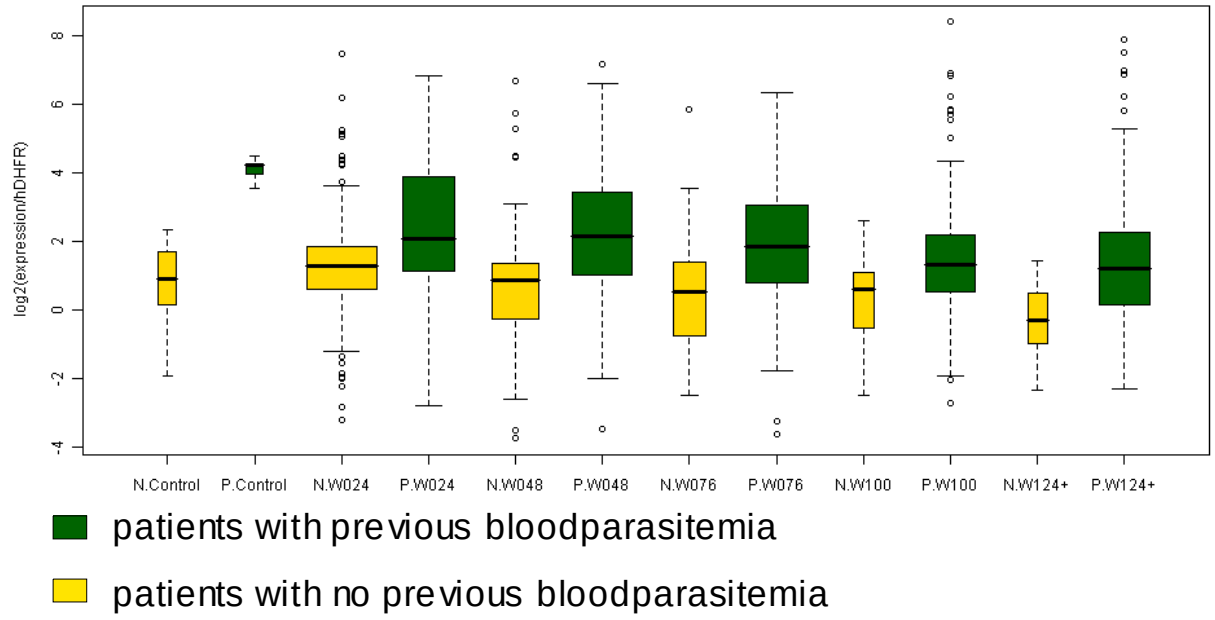

Figure 1C: Reactivity of patient sera against PFD1120c

Unfortunately, the presence of antibodies against PFD1120c did not correlate with protection against severe anemia (Fig 1D), suggesting that this protein is a marker for malaria experience and not the target of a protective immune response.

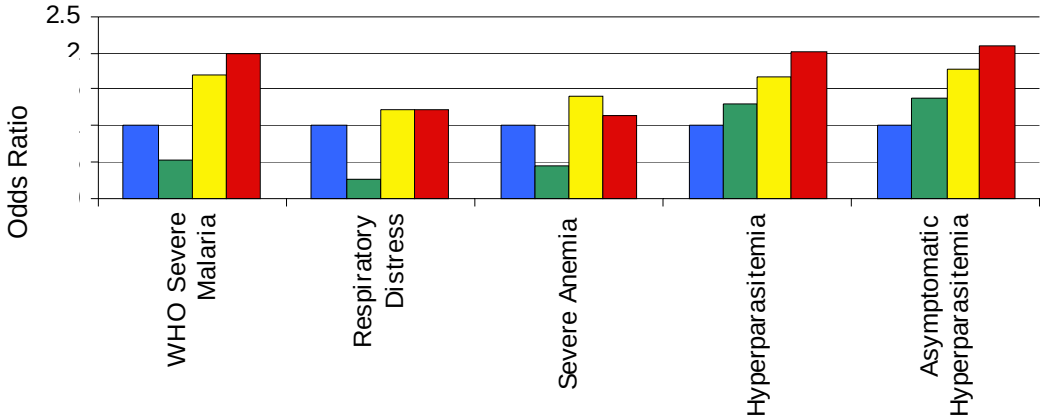

Figure 1D: Risk of malaria outcomes by level of PFD1120c antibodies. Odds ratios are presented for second, third, and forth quartile of antibody level versus the first quartile.

As listed in Table 2, other candidates originally identified by microarray as up-regulated in parasite samples isolated from anemic patients when compared to their non-anemic peers were either not confirmed by qPCR or are being pursued by the indicated methods. We sought in particular to identify var genes that appear to be more highly expressed among parasites isolated from children with clinical symptoms of severe anemia, since the PfEMP1 proteins encoded by these variable gene family are known to be localized on the surface of the infected red blood cells and to have a role in adhesion to human endothelia and immune system evasion, From the Morogoro/genomic tiling array dataset, the top two expressed PfEMP1-like genes were PFB1045w and MAL7P1.186. These genes are truncated in the reference sequenced strain 3D7, and the region identified by our tiling microarray correspond to the cytoplasmic tail of var genes PFF0020c and PFI1820w. We are currently pursuing these candidates further.

In addition to being useful for the identification of malaria vaccine candidates, the data generated from the analysis of field sample RNA by genomic tiling microarrays has allowed us to better define the 5' and 3' untranslated regions (UTRs) of *P. falciparum* genes, which are poorly described, and has resulted in the identification of potentially novel transcriptional units that do not correspond to genes predicted in the latest genome version. A subset of these are being validated by qPCR, and the resulting data will be summarized with the goal of publishing a manuscript describing the use of genomic tiling arrays to define transcriptional units in *P. falciparum.*

Finally, we have designed a new tiling array based on the data obtained from the 20 arrays processed with children field samples (as well as the results from a secondset of slides from a parallel project in the lab). We selected a subset of informative probes from the original genomic tiling design to include in a novel, 12-plex format with a total of 135,000 probes. The new design includes ~75,000 probes for unique *P. falciparum* proteins that capture 98% of the information yielded by the full set of 385,000 probes used previously. In addition, we have enriched the array for the purpose of detecting variable surface antigens (vsa, i.e. var, rifins and stevors) from the reference 3D7 strain, as well as strains IT4, HB3 and Dd2. We also included 5' UTR probes, probes for regions that do not correspond to predicted genes but were identified in our initial 20 slides as being potentially transcribed, and probes for human genes that have been involved in immunity. This new design, which allows the analysis of 12 samples per slide plus our recent purchase of a hybridization station will allow us to process these slides in-house at a greatly reduced cost per sample.

In addition to the different microarray approaches described above, more recently we have applied the new technique of RNA sequencing to identify genes up-regulated in anemic samples. In collaboration with Merck/Rosetta, we analyzed the gene expression profile of *P. falciparum* samples by means of the Illumina platform. The method includes a cDNA generation step that preserves transcript directionality, and has been modified by our collaborators to exclude human globin and rRNA, and after deep sequencing yields a very high number of ~36 bp reads, which can be assembled and/or aligned to an existing genome. In contrast to microarray analysis, RNAsequencing is digital and is not limited by saturation and cross-hybridization issues, and in addition, the interpretation of the resulting data does not depend on previous knowledge of the genome of the organism being sequenced. These features make the approach ideal for the analysis of field samples where high variability is expected, in particular for variable surface antigens encoded by *P. falciparum* gene families var, rifins and stevors. The Pilot phase of this project included the analysis of an unsynchronized, lab-adapted sample of reference strain 3D7 (using uninfected

human blood as a negative control), and revealed that the majority of the reads obtained by this method could be aligned unambiguously, and that only a small percentage % of the reads from the infected blood sample aligned to the human genome (and vice versa, see Table 3).

	Blood	Infected blood
matches both malaria + human	2,127	18,419
matches redundantly	1,373,380	1,074,480
matches neither anywhere	1,482,052	1,639,377
malaria only	8,329	1,892,418
human only	3,210,682	220,620
sum	6,076,570	4,845,314

Table 3: Number of sequencing reads for uninfected blood and blood with cultured parasites

To determine if the technology could be used to analyze relative expression levels between samples, we conducted a second experiment in which we compared gene expression between pools of ~8 maternal vs. children lab adapted samples. 65% of the reads could be unambiguously assigned, and of those >99% aligned to the parasite's genome. The remaining 35% of the reads have not been analyzed deeply yet, but might correspond to vsa that differ between field samples and 3D7. While the majority of the genes showed no difference in expression among the two samples (R=0.97, Fig. 2), a subset of genes displayed a clear differential regulation between the maternal and children pools.

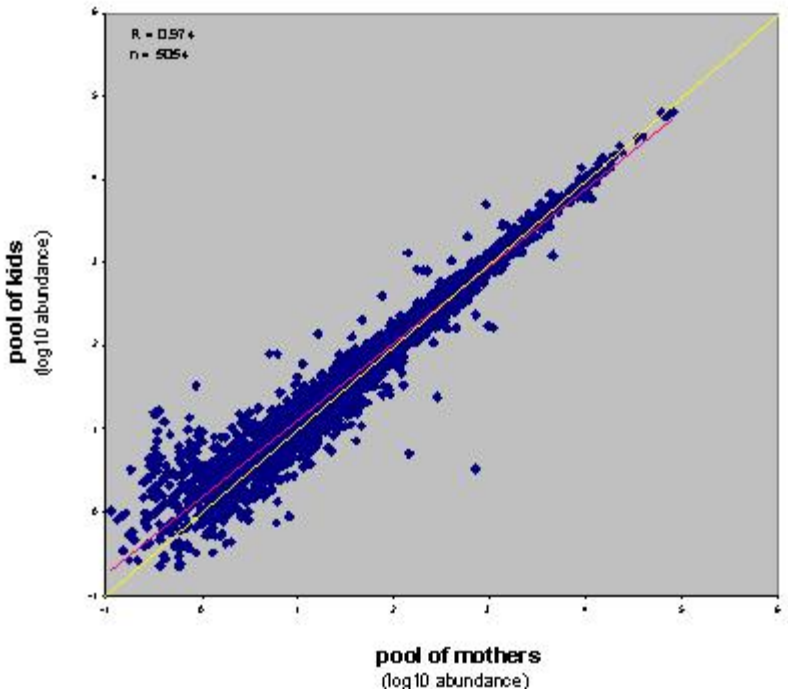

Figure 2: *P. falciparum* transcript levels in lab-adapted human blood samples

The data was compared to a published transcriptome study from our group which had identified 7 genes up-regulated in peripheral blood samples of mothers vs. children (Francis et al., 2007). Importantly, not only did the best characterized pregnancy malaria gene,

PFL0030c/var2csa show a distinct pattern of up-regulation in maternal samples, but 4 other genes reported to be up-regulated in maternal peripheral blood by microarray analysis are found in the top 1% differentially expressed genes when the maternal and children pools are compared. This high level of agreement indicates the ability of the RNA sequencing technique to identify differentially regulated genes, and additionally suggests that it has the potential to identify a large number of putative candidates beyond those that were identified by microarray-based approaches.

The experiment has also demonstrated that RNA sequencing can be used to analyze parasite lines that differ from the published strain to identify differentially regulated genes. With the ultimate goal of identifying genes upregulated in field parasites isolated from anemic episodes, we are currently optimizing the approach by analyzing pools of field isolates to determine the influence of having a larger proportion of human RNA in the sample, and also to identify the minimal amount of material that can be utilized,

In addition, this project has yielded unexpected discoveries, such as the identification of alternative splicing events in 144 genes, a process that has only been described for a handful of falciparum genes to date.

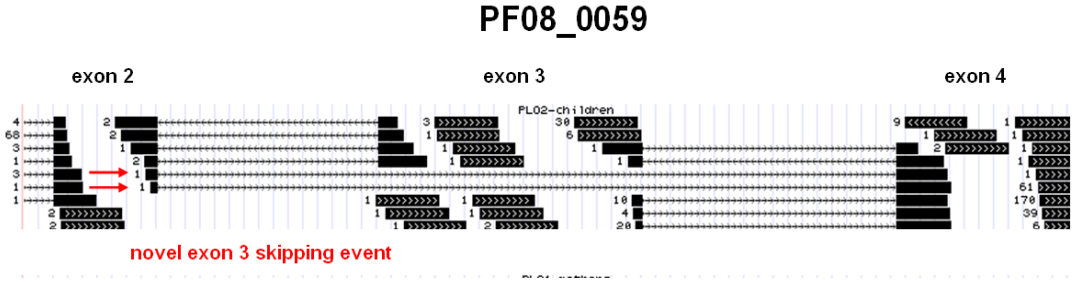

Figure 3: An example of alternative splicing found by RNA sequencing. Red arrows point to sequencing reads that span exons 2 and 4, but not exon 3.

Finally, we have initiated a third approach for the identification of potential candidates for a severe anemia vaccine, based on phage display technology. This approach does not rely on the quantification of steady state RNA levels, but instead directly looks at proteins expressed by field samples and analyzes the reactivity of sera samples to these candidates. We have generated cDNA libraries using lab-adapted children field samples, and are currently screening them to select proteins that bind common host receptors, including CD36, CSA and ICAM1. Our ultimate goal is to use RNA from field samples to generate libraries that will be screened with sera from exposed individuals in a high-throughput platform, in order to identify parasite proteins that are recognized by sera from individuals with particular clinical features.

Proteomics Studies of Severe Anemia Parasites

The overall goal of this project was to characterize the proteome of parasites collected from children with malarial anemia as a means to identify potential targets that can be further evaluated as vaccine candidates. One of the major challenges in this study was the limited amount of parasite proteins available for proteomics studies from anemia cases. Final analysis included four parasites collected from children with moderate anemia (hemoglobin 6-8 gr/dL) and one case of severe malarial anemia (hemoglobin <5 g/dL). The proteome profile of these parasites was compared to that of parasites collected from hyperparasitemia cases, another common malaria syndrome in the study area (23 cases).

To identify uniquely expressed proteins we compared the frequency of protein expression within a group (anemia *vs.* hyperparasitemia) that will allow identifying unique or preferentially expressed proteins in parasites causing anemia. The table below lists 16 proteins that were either expressed by parasites from anemia cases only or proteins that were detected in higher proportion of parasites from anemia cases.

Among the 16 proteins, 3 contained a *Plasmodium* exported element (pexel) and 10 proteins predicted to contain a signal peptide (SP) or transmembrane domain (TM). These results are currently undergoing validation for preferential expression by qRTPCR as a complementary accurate approach to validate proteomics data.

Protein ID	% anemia	% hyperparasitemia	Pexel	SP	TM
PF14_0714	60	0	N	Y	Y
MAL13P1.110	40	0	N	Y	Y
PFL2355w	60	4.3	N	N	N
PF10_0361	60	13	N	N	N
PFA0410w	40	8.7	N	N	N
PFF0220w	40	8.7	N	N	N
PF11_0364	60	17.4	N	Y	N
PFD0095c	40	13	Y	Y	Y
MAL13P1.237	60	21.7	N	N	N
MAL13P1.226	40	17.4	N	N	Y
MAL13P1.233	40	17.4	N	N	N
PF11_0506	60	34.7	N	N	Y
PF13_0192	40	26	N	N	Y
PFE0050w	40	26	Y	N	Y
MAL7P1.171	60	39	Y	N	Y
PF08_0137	20	4.3	N	N	Y

Table 4: Hypothetical proteins preferentially expressed by parasites associated with anemia

KEY RESEARCH ACCOMPLISHMENTS: Below is a list of key research accomplishments emanating from this research:

- Ongoing regulatory compliance with human and animal protocols, with annual continuing review approvals obtained.
- Identified several known surface protein genes (called *var* genes) that are upregulated in parasites causing anemia
- Identified several hypothetical protein genes that are upregulated in parasites from anemic patients that would also make good vaccine antigen candidates
- Established cell-free protein expression in our laboratory using ENDEXT technology to prepare recombinant proteins for serosurveys
- Improved the quality of our oligonucleotide spotted microarrays and implemented quality control analyses to ensure quality and consistency of slides between different prints
- Improved RNA stabilization and extraction assays
- Completed microarray studies of parasites causing severe anemia in Tanzania
- Validated results obtained from our in-house spotted arrays using tiling arrays from Nimblegen
- Applied the new technique of RNA sequencing to identify genes up-regulated in anemic samples
- Initiated a third approach for the identification of potential candidates for a severe anemia vaccine, based on phage display technology

REPORTABLE OUTCOMES:

Publications

1. Duffy PE, Fried M. 2006. Red blood cells that do and red blood cells that don't: how to resist a persistent parasite. Trends Parasitol, 22(3):99-101
2. Kappe SHI, Duffy PE. 2006. Malaria liver stage culture: *in vitro veritas?* Am J Trop Med Hyg, 74(5):706-7
3. Duffy PE, Mutabingwa TK. 2006. Artemisinin combination therapies. Lancet, 367(9528):2037-9.
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5. A Muehlenbachs*, TK Mutabingwa, M Fried, Duffy PE. 2007. An unusual presentation of placental malaria: a single persisting nidus of sequestered parasites. Human Pathol, 38(3):520-3. Epub 2007 Jan 19.
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9. Francis SE, Malkov VA, Oleinikov AV, Rossnagle E, Wendler JP*, Mutabingwa TK, Fried M, Duffy PE. 2007. Six genes are preferentially expressed by the circulating and sequestered forms of *Plasmodium falciparum* parasites that infect pregnant women. Infect Immun, 75:4838-50.
10. Duffy PE. 2007. *Plasmodium* in the placenta: parasites, parity, protection, prevention, and possibly pre-eclampsia. Parasitol, 134:1877-81.
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15. Kabyemela ER*, Fried M, Kurtis JD, Mutabingwa TK, Duffy PE. 2008. Fetal responses during placental malaria modify the risk of low birth weight. Infect Immun. 76(4):1527-34.
16. Harrington W*, Duffy PE. 2008. Congenital malaria: rare but potentially fatal. Ped Health. 2(2):235-48.
17. Kabyemela ER*, Fried M, Kurtis JD, Mutabingwa TK, Duffy PE. 2008. Decreased Susceptibility to *Plasmodium falciparum* Infection in Pregnant Women with Iron Deficiency. J Infect Dis. 198(2):163-6.
18. Kabyemela ER*, Muehlenbachs A*, Fried M, Kurtis JD, Mutabingwa TK, Duffy PE. 2008. Maternal peripheral blood level of IL-10 as a marker for inflammatory placental malaria. Malar J. 7:26.

19. Muehlenbachs A*, Fried M, Lachowitzer J, Mutabingwa TK, Duffy PE. 2008. Natural selection of FLT1 alleles and their association with malaria resistance in utero. Proc Natl Acad Sci USA. 105(38):14488-91.
20. Oleinikov AV, Amos E, Frye IT, Rossnagle E, Mutabingwa TK, Fried M, Duffy PE. 2008. High throughput functional assays of the variant antigen PfEMP1 reveal a single domain in the 3D7 *P. falciparum* genome that binds ICAM1 with high affinity and is targeted by naturally acquired neutralizing antibody. PLoS Pathogens. In press.
21. Vignali M, Speake C*, Duffy PE. 2008. From genomics to interventions for malaria: Recent advances in malaria functional genomics. Genome Biology. Submitted.
22. Speake C*, Duffy PE. 2008. Antigens for Pre- erythrocytic Malaria Vaccines: Building on Success. Parasite Immunology. Submitted.
23. Harrington W*, Mutabingwa TK, Muehlenbachs A*, Sorensen B, Bolla MC, Fried M, and Duffy PE. Partially Effective Antimalarials Exacerbate *Plasmodium falciparum* Malaria during Pregnancy. Submitted.

Presentations

1. Invited Speaker, “Malaria vaccines”, Science & Technology Roundtable. Seattle, Washington. 10 February 2006
2. Seminar Speaker, “Tropical Diseases Research”, Seattle Pacific University, Natural Sciences Seminar. 13 February 2006.
3. Symposium Speaker. “Malaria Pathogenesis and the Parasite Genome.” Keystone Symposia, Malaria: Functional Genomics to Biology to Medicine. Taos, New Mexico. 28 February – 5 March 2006
4. Seminar Speaker. “Malaria at the mother-child interface: epidemiology, pathogenesis, interventions.” NIH Seminar Series. Bethesda, Maryland. 28 March 2006.
5. Invited Speaker. President’s Malaria Initiative: Research into Practice Meeting. Kampala, Uganda. 25-28 April 2006.
6. Medicine Grand Rounds Speaker. “Malaria Pathogenesis and a Vaccine.” University of Tennessee, Memphis. 21 June 2006.
7. Keynote Speaker. “Pregnancy Malaria: Two Parasites and an Inflamed Host.” ICOPA XI. Glasgow, Scotland, United Kingdom. 6-11 August, 2006.
8. Seminar speaker. "Malaria during Pregnancy: Epidemiology, pathogenesis, immunology." Statistical Center for HIV/AIDS Research and Prevention, Fred Hutchinson Cancer Research Center. 11 September 2006.
9. Symposium Speaker. “Field studies in Africa.” GCGH All Grantee Meeting, Washington, D.C. 4-6 October 2006.
10. Symposium Speaker. “Parasite diversity and the spectrum of disease due to P. falciparum.” ASTMH 55th Annual Meeting, Atlanta, Georgia, 12-16 November, 2006.
11. Invited Speaker. “Pathological effects of pregnancy malaria on mother and offspring.” Israeli Society for Parasitology, Protozoology, and Tropical Diseases Annual Meeting and Severe Malaria Workshop, Maale Hahamisha, Israel. 13-14 December 2006.
12. Symposium Speaker. “Malaria in Pregnancy.” Shoklo Malaria Research Unit 20 Years Anniversary Seminar, Mae Sot, Thailand. 28 December 2006.
13. Symposium Speaker. “Functional genomics tools and field studies of *Plasmodium falciparum*.” Malaria Parasite Diversity Meeting, Hinxton, Cambridgeshire. 20-22 January 2007.
14. Invited Speaker. “Malaria control strategies: The human immune system.” Western Regional International Health Conference, Seattle, Washington. 16-18 February 2007.
15. Symposium Speaker. “Malaria Control.” Whitehead Institute annual press conference, Cambridge, MA. 23 April 2007.

16. Seminar Speaker. “Malaria at the mother-child interface.” Albert Einstein College of Medicine, Bronx, NY. 9 May 2007.
17. Co-organizer. Symposium: “The molecular background to severe and complicated malaria.” Karolinska Institutet. Stockholm, Sweden. 14-16 June 2007.
18. Co-organizer. East African Regional Training Workshop: “Genomic Studies of Protozoan Pathogens.” Sokoine University. Morogoro, Tanzania. 1-14 July 2007.
19. Symposium Speaker. “Malaria in Pregnancy.” Gordon Conference, Oxford, UK. 9-14 September 2007.
20. Symposium Speaker. “Protective immunity to severe malaria in African children.” Keystone Symposium, Challenges of Global Vaccine Development, Cape Town, South Africa. 8-12 October 2007.
21. Hematology Grand Rounds Speaker. “Malaria pathogenesis at the mother-child interface.” University of Washington. Seattle, WA. 18 January 2008.
22. Symposium Speaker. “Fetal responses during placental malaria.” MAM Conference. Melbourne, Australia. 3-7 February 2008.
23. Invited Speaker. Institute for Health Metrics & Evaluation. Seattle, WA. 13 March 2008.
24. Invited Speaker. Malaria Next Steps. London, UK. 8-9 April 2008.
25. Keynote Speaker. BioMalPar 4th annual meeting on the "Biology and Pathology of the Malaria Parasite.” Heidelberg, Germany. 14-16 April 2008.
26. Symposium Speaker. Keystone Meeting, Malaria: Immunology, Pathogenesis and Vaccine Perspectives. Alpbach, Austria. 8-13 June 2008.
27. Symposium Speaker. “Vaccine Development for Blood Stage Malaria at SBRI.” 6th Matsuyama International Symposium on Cell-free Sciences. Matsuyama, Japan. 26-27 September 2008.
28. Symposium Speaker. “Progress on a Vaccine to Protect Pregnant Women from *Plasmodium falciparum.*” 17th International Congress for Tropical Medicine and Malaria. Jeju Island, Korea. 29 September – 3 October 2008.
29. Symposium Speaker. “Malaria in the Mother Influences Malaria Outcomes of her Offspring During Infancy.” 17th International Congress for Tropical Medicine and Malaria. Jeju Island, Korea. 29 September – 3 October 2008.
30. Invited Speaker. “Protective immunity against severe malaria in young children.” 4th Annual Grand Challenges in Global Health Meeting. Bangkok, Thailand. 19-22 October 2008.
31. Seminar Speaker. “Malaria pathogenesis in pregnant women and young children.” Institute for Systems Biology. Seattle, WA. 29 October 2008.
32. Invited Expert and Speaker. MalERA Vaccine Consultative Group Meeting. Montreux, Switzerland. 22-24 November, 2008.
33. Symposium Speaker. “Antigens and the prospects for accelerating antigen discovery.” ASTMH Symposium. New Orleans. 7-11 December, 2008.
34. Symposium Speaker. “Measuring and interfering with infected red blood cell adhesion.” ASTMH Symposium. New Orleans. 7-11 December, 2008.
35. Featured Speaker. "Pregnancy Malaria Research in Africa.” ASCB Meeting. San Francisco. 13-14 December, 2008.

CONCLUSION: Malaria is the leading infectious disease threat to US troops, and parasites have emerged that are resistant to all existing drugs used for chemoprophylaxis against malaria.

Our project has confirmed our hypothesis that specific genes and proteins are upregulated in the parasites that cause malarial anemia. These include 26 genes that were significantly

upregulated in DNA microarray studies of anemia parasites, including two *var* genes known to encode surface proteins of malaria parasites, and 16 proteins that were present in greater abundance in membrane extracts from of infected erythrocytes from children with malarial anemia. We have established the methodologies required to validate these genes and proteins for their relationship to malarial anemia, and to relate immune responses against these to protection from severe malaria. Our early results indicate that a subset of these genes identified by microarrays can be confirmed as upregulated in malarial anemia parasites, according to qPCR studies. These validation studies remain ongoing, and may yield a candidate antigen with the features desired in a potential vaccine: a surface antigen preferentially expressed by the parasites causing malarial anemia and targeted by functional antibody that correlates with protection from anemia during malaria. Our studies have also integrated novel and cutting-edge additional approaches for identifying candidate antigens, such as high throughput sequencing, and phage display of malaria polypeptides to allow subtractive selection of proteins associated with protective antibody using sera from endemic areas.

The overall result of these studies has been to establish a limited subset of malaria genes/proteins as potential antigens for a malarial anemia vaccine, and to establish the tools that will allow us to validate these as vaccine candidates in the immediate future. A vaccine that prevented malarial anemia would be an important tool to protect US troops from severe sequelae of malaria during infections incurred while deployed in tropical areas.

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Richa Chaturvedi – Research Technician
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Malaria: progress, perils, and prospects for eradication

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There are still approximately 500 million cases of malaria and 1 million deaths from malaria each year. Yet recently, malaria incidence has been dramatically reduced in some parts of Africa by increasing deployment of anti-mosquito measures and new artemisinin-containing treatments, prompting renewed calls for global eradication. However, treatment and mosquito control currently depend on too few compounds and thus are vulnerable to the emergence of compound-resistant parasites and mosquitoes. As discussed in this Review, new drugs, vaccines, and insecticides, as well as improved surveillance methods, are research priorities. Insights into parasite biology, human immunity, and vector behavior will guide efforts to translate parasite and mosquito genome sequences into novel interventions.

Introduction

More than 2 billion people are at risk of malaria (1), which primarily affects poor populations in tropical and subtropical areas, where the temperature and rainfall are most suitable for the development of the malaria-causing *Plasmodium* parasites in *Anopheles* mosquitoes. Malaria once occurred widely in temperate areas, including Western Europe and the United States, but it receded with economic development and public health measures. The disease was finally eliminated in the US between 1947 and 1951 through a campaign that included household spraying of the residual insecticide dichloro-diphenyl-trichloroethane (DDT) throughout the southeastern states (2).

The Global Malaria Eradication Programme was launched by the WHO in 1955 (3) and depended on two key tools: chloroquine for treatment and prevention and DDT for vector control. Implementation of these tools had a substantial impact in some areas, particularly areas with relatively low transmission rates, such as India and Sri Lanka (3). Despite these successes, the campaign foundered in the face of lost political will and the emergence of chloroquine-resistant *Plasmodium* parasites and DDT-resistant *Anopheles* mosquitoes. Global eradication was officially abandoned as a goal in 1972 (4). Furthermore, the campaign never attempted to eradicate malaria in most parts of Africa, where malaria transmission is intense.

Since the Global Malaria Eradication Programme ended, the burden of malaria has increased substantially in many parts of the world, although in some countries (e.g., Thailand), transmission has continued to decline in parallel with economic development, improved health infrastructure, and continued anti-vector measures (5). The resurgence of malaria was sometimes dramatic, including epidemics in Sri Lanka in 1968–1969 and in Madagascar in 1987–1988 (6). Childhood deaths in Africa due to malaria climbed relentlessly as chloroquine-resistant *Plasmodium* parasites spread across the continent (7). The rapid emergence of *Plasmodium* parasites resistant to sulfadoxine-pyrimethamine soon after this drug replaced chloroquine as first-line therapy in many parts of Africa prompted a group of leading malaria experts to warn of an impending disaster in Africa (8).

In response to this dire situation, the global community is now taking steps to deliver more effective interventions throughout Africa, including drug combinations with an artemisinin derivative and anti-vector measures. The dramatic success of these measures in a few specific areas, such as KwaZulu-Natal in South Africa (9), Eritrea (10), and the Tanzanian island of Zanzibar (11), has inspired a new call for global eradication. Achieving this ambitious goal depends on the development of new tools to treat, prevent, and monitor malaria. Further, the recent availability of genome sequences for humans, *Anopheles* mosquitoes, and *Plasmodium* parasites has raised hopes for new interventions. As this Review describes, we need a deeper understanding of parasite biology, human immunity, and vector behavior to maximally exploit genomic data for the discovery of new interventions, and these discovery efforts must be balanced against more applied research that addresses immediate priorities such as optimal implementation and protection of existing treatment and control tools.

The life cycle of *Plasmodium* parasites and targets for intervention

Among the four *Plasmodium* species that cause malaria in humans, *Plasmodium falciparum* is the most virulent. This species causes the vast majority of deaths from malaria and is also distinguished by its ability to bind to endothelium during the blood stage of the infection (Figure 1) and to sequester in organs, including the brain.

Nonstandard abbreviations used: ACT, artemisinin-based combination therapy; CSA, chondroitin sulfate A; CSP, circumsporozoite protein; DDT, dichloro-diphenyl-trichloroethane; GPI, glycosylphosphatidylinositol; IE, infected erythrocyte; IRS, indoor residual spraying; ITN, insecticide-treated bednet; PV, parasitophorous vacuole.

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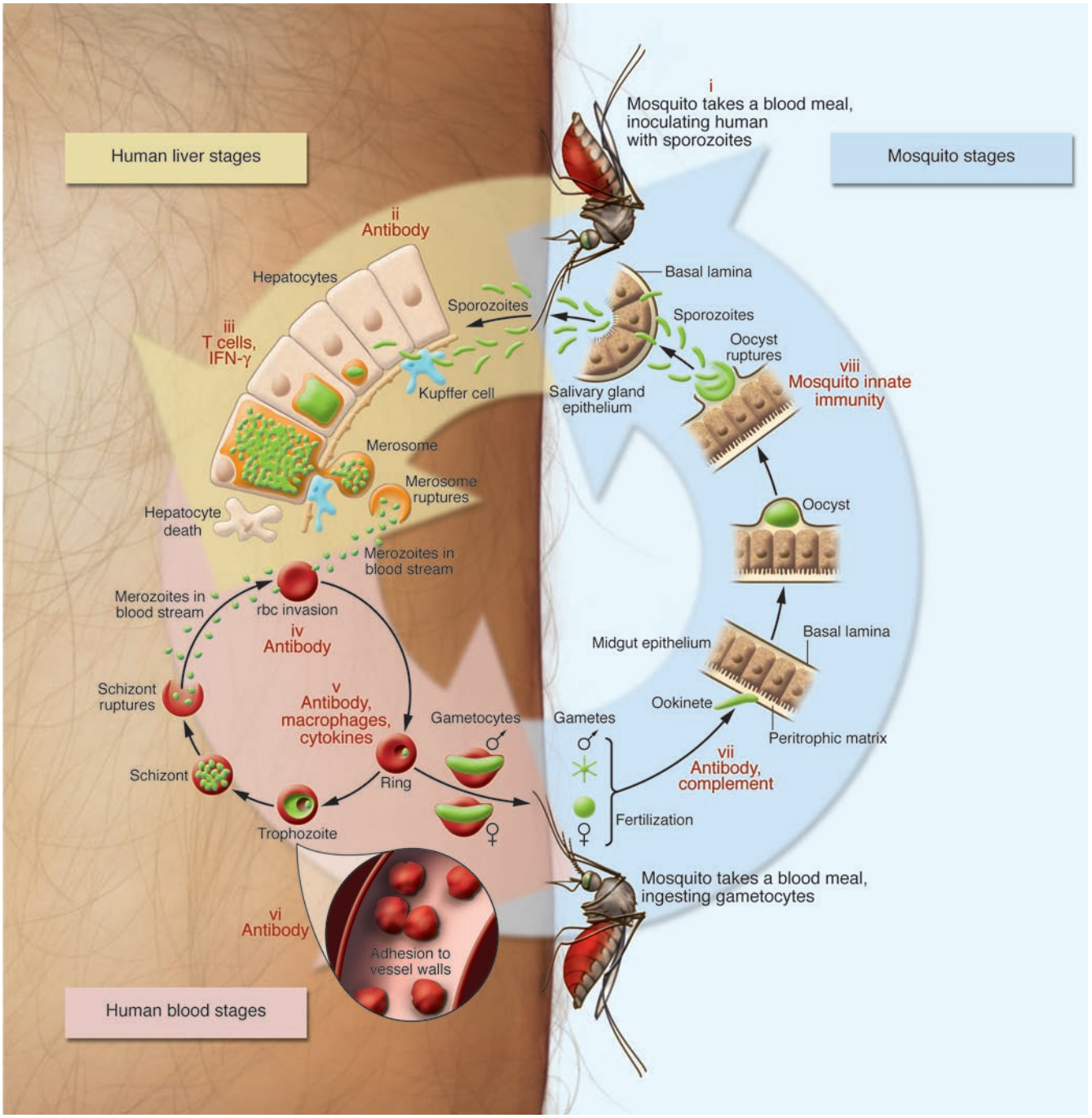

Figure 1
The life cycle of malaria-causing *Plasmodium* parasites. The *Plasmodium* life cycle comprises numerous transitions and stages, and any of these can be targeted by host immune responses. Upon inoculation by an *Anopheles* mosquito into the human dermis, elongated motile sporozoites must evade antibodies to (i) access blood vessels in the skin and then (ii) transit through liver macrophages and hepatocytes to initiate liver stage infection. Intrahepatocytic parasites (iii) are susceptible to CTLs. After approximately one week, infected hepatocytes rupture and release merozoites as aggregates called merosomes that might allow merozoites to (iv) evade antibodies and invade erythrocytes. Intraerythrocytic parasites (v) are susceptible to opsonizing antibodies and macrophages, and cytokine responses have been related to both protection and disease during this stage of infection. Antibodies that block (vi) binding of *P. falciparum*–infected erythrocytes to endothelium might prevent disease and control parasitemia. Human antibodies specific for (vii) sexual stage parasites are taken up by mosquitoes during the blood meal and can block transmission to mosquitoes, although these might require complement for parasite killing. *Anopheles* mosquito innate immune responses can also kill parasites during early (vii) or late (viii) sporogonic stages and lead to refractoriness to infection.

Potential end points for assessing the impact of an antimalarial intervention

Nonpregnant adults and children[A]
- All causes of mortality
- Malaria-specific mortality
- Hospital admissions with severe malaria
- Laboratory-confirmed clinical attacks of malaria
- Prevalence of malaria parasitemia in the community
- Prevalence of anemia in the community

Pregnant women
- Maternal mortality
- Prevalence of anemia
- Incidence of low birth weight

Vector mosquitoes
- Numbers
- Infection rate

[A]In highly endemic areas, the main burden of malaria is in children, and most surveys focus on this age group.

Plasmodium vivax is less deadly but highly disabling; it is common in tropical areas outside Africa (most Africans lack the Duffy blood group antigen that is expressed on the surface of erythrocytes and is a necessary receptor for *P. vivax* invasion of these cells). The ability of *P. vivax* and also *Plasmodium ovale* to remain dormant for months as hypnozoites in the liver makes infection with these parasites difficult to eradicate. *Plasmodium malariae* does not form hypnozoites, but it can persist for decades as an asymptomatic blood stage infection. A fifth species, *Plasmodium knowlesi*, which was originally described as a malaria parasite of long-tailed macaque monkeys, also naturally infects humans in some areas, such as Malaysia (12).

Infection of the human host with a *Plasmodium* parasite begins with the bite of an infected *Anopheles* mosquito that inoculates the individual with sporozoites (Figure 1). These motile forms of the parasite rapidly access the blood stream and then the liver, where they invade hepatocytes. The asymptomatic liver stage of infection lasts about 6 days, with each sporozoite yielding tens of thousands of merozoites that then invade and develop within erythrocytes. The blood stages of infection include asexual forms of the parasite that undergo repeated cycles of multiplication as well as male and female sexual forms, called gametocytes, that await ingestion by mosquitoes before developing further. Asexual blood stage parasites produce 8–20 new merozoites every 48 hours (or 72 hours for *P. malariae*), causing parasite numbers to rise rapidly to levels as high as 10^{13} per host. The asexual stages are pathogenic, and infected individuals can present with diverse sequelae affecting different organ systems. Sexual stage parasites are nonpathogenic but are transmissible to the *Anopheles* vector, where they recombine during a brief period of diploidy and generate genetically distinct sporozoites (13). The mosquito becomes infectious to its next blood meal donor approximately two weeks after ingesting gametocytes, a time frame that is influenced by the external temperature. Development of *P. vivax* within the mosquito can occur at a lower environmental temperature than that required for the development of *P. falciparum*, explaining the preponderance of *P. vivax* infections outside tropical and subtropical regions.

During its peripatetic existence, the unicellular malaria-causing parasite uses a toolkit of more than 5,000 genes (14) to undergo dramatic metamorphoses that are suited to the numerous environments and barriers it encounters. These changes include the development, at different points in its life cycle, of motile, invasive, encysted, intracellular, sexual, and dormant forms. These distinct forms of the parasite help enable it to complete its full life cycle (Figure 1), during which it must passage through the mosquito midgut and salivary glands; localize and penetrate skin vessels; perforate and traverse macrophages and several hepatocytes prior to enveloping itself in an intrahepatocytic vacuole; and attach to and reorient itself on the surface of erythrocytes prior to invasion.

Each of the developmental stages discussed above represents a potential target at which the life cycle can be interrupted. Vaccines, drugs, and anti-vector measures are being developed to prevent infection, disease, and transmission. Despite numerous potential targets, the most widely used old compound (quinine, isolated from cinchona bark in 1820) and the best new compound (artemisinin, purified from *Artemisia annua* in 1972) for treatment are both derived from ancient herbal therapies. Further, progress with developing a vaccine is incomplete. These limitations stem, in part, from the fact that since its discovery in 1880 (15), the parasite has been slow to reveal its secrets, including its metabolic pathways and its antigens that are targeted by protective immunity. However, recent advances in determining the genome sequences for humans, *Anopheles* mosquitoes, and *Plasmodium* parasites have raised hopes that developing new interventions might be feasible.

Epidemiology and clinical features

Global disease burden and surveillance. Efforts to control malaria are being made on a scale not seen for fifty years. However, the long-term sustainability of this effort depends on demonstrating a beneficial impact on health and development. This requires that the distribution and burden of malaria be determined before and after the initiation of interventions — data that are hard to collect. Indeed, most malaria-endemic countries, particularly those in sub-Saharan Africa, have weak health information systems and civil registries, and the consequences of a malaria infection are varied, meaning that many indicators are used to measure the impact of an intervention (see *Potential end points for assessing the impact of an antimalarial intervention*). Malaria is such a common cause of childhood death that successful interventions are likely to have a discernible impact on the overall childhood mortality rate in endemic areas. Overall mortality can be measured either through indirect demographic techniques or directly in sites with continuous demographic surveillance systems. In contrast, malaria-specific mortality is much more difficult to document because most deaths from malaria occur at home. Malaria morbidity requires either the use of health facilities as sentinel sites or regularly conducted community-based surveys.

Despite the difficulties, considerable progress has been made in defining the global distribution of malaria (16, 17) and its burden. Because the clinical diagnosis of malaria is imprecise, estimates of the burden of malaria that rely upon clinical data without laboratory support are unreliable. However, improved regional and global estimates of the malaria burden have used accurate data collected at a limited number of areas with well-defined geographical, entomological, and population characteristics, which are then extrapolated to other areas with similar characteristics and known populations. Studies of this kind suggest that malaria directly

causes just under 1 million deaths and at least 500 million clinical cases each year (1, 18). Furthermore, malaria in pregnancy contributes to a substantial number of maternal deaths as well as infant deaths resulting from low birth weight (19).

Transmission of malaria-causing parasites is typically infrequent but also unstable (i.e., transmission varies in prevalence and is prone to change) in substantial areas of Southeast Asia and South America and might become more unstable in Africa as disease control improves. Because levels of immunity are also low, areas of unstable transmission are prone to epidemics, during which mortality and morbidity can be very high. Research that improves the prediction of epidemics is therefore critical. Climate modeling can give long-range warnings of heavy rainfalls, and hence of increased mosquito breeding and risk of malaria (20). Active surveillance at district health centers can detect an early increase in cases and allow appropriate control measures to be put in place before a large epidemic explodes (21). As malaria control improves, surveillance will become necessary to identify persistent and new foci of infection as well as localized areas where control measures are not working. Sensitive PCR techniques for detecting asexual (22) and sexual stage infections (23) as well as new serological methods (24) might be helpful at this stage of malaria control.

Diagnosis and clinical spectrum of disease. Clinical diagnosis of malaria is difficult, and misdiagnosis is frequent when laboratory confirmation is not available or is disregarded by doctors anxious to identify a treatable cause of illness. Microscopy remains an important tool for diagnosis, but laboratory diagnosis in clinics without microscopy has now become possible through the development of rapid diagnostic tests (25). Some tests, however, deteriorate under tropical conditions and can give false results (26). Furthermore, health care staff might still overtreat for malaria even when rapid tests are available (27), possibly because they lack facilities to identify other causes of fever and because malaria symptoms overlap with those of many illnesses. Finding ways to change the attitudes of health care providers and the perceptions of patients as to what they should receive at the clinic is an important applied research priority.

Uncomplicated malaria usually presents with fever and nonspecific symptoms, such as vomiting and/or diarrhea, a clinical picture that resembles that of many other childhood infectious diseases. In adults, severe malaria caused by *P. falciparum* is characterized by multiorgan damage, including renal failure. This is uncommon in children with severe malaria, who usually present with prostration, respiratory distress, severe anemia, and/or cerebral malaria. Each of these clinical presentations of malaria probably represents a complex of conditions, each with their individual pathogenesis, complicating the effort to develop broadly effective adjunctive therapies. Additional abnormalities, such as hypoglycemia and acidosis, can complicate and/or contribute to severe malaria.

What determines the pattern of severe malaria in an individual case is not fully understood. Genetic factors are important (28), and both the age of the patient and the intensity of transmission in the community influence susceptibility to cerebral malaria and severe anemia (29). Cerebral malaria is a more common presentation of severe malaria where transmission intensity is low, whereas severe anemia predominates where transmission intensity is high. Retinal changes occur in many patients with severe malaria, and a specific pathology was recently described that might aid diagnosis (30). Children with severe malaria rarely present with the classical features of circulatory shock, but recent studies have suggested, controversially, that many have hypovolemia that contributes to their acidosis (31, 32).

Malaria can interact with other infectious diseases to modify the susceptibility and/or severity of either disease. For example, solid evidence now indicates that infection with HIV increases the risk of uncomplicated and severe malaria (33). Conversely, malaria causes a transitory increase in viral load (34), and this could promote HIV transmission. In pregnant women, malaria and infection with HIV interact to cause low birth weight, and HIV impairs the efficacy of drugs used for malaria prevention (35). Coinfection with helminths seems to protect against severe malaria in some, but not all, areas, which might indicate that the interaction is site specific and determined by local patterns of malaria and helminth infections (36, 37). Finally, a marked percentage of African children with severe malaria are increasingly recognized to have associated bacteremia, and these patients might have increased mortality (38). Infection with nontyphoidal *Salmonella* spp. is an important complication of severe malaria anemia, but the mechanism of this association is not understood (39).

Parasite biology, drugs, and resistance

The discovery of new agents to prevent or treat malaria has benefited from the sequencing of the parasite genome (14) and the development of improved tools for functional genomics (40–43), yet this area of research remains limited by our incomplete knowledge of parasite biology. In the case of the asexual blood stage parasites, research has identified several processes, such as hemoglobin degradation and heme detoxification, folate biosynthesis, and protein synthesis in the apicoplast, as effective targets for therapeutic intervention (44). An expansion of this research effort is critical to defining the pathways that are most suitable for intervention, to validating candidate drug targets (Figure 2), and to identifying chemically tractable inhibitors for drug development. The discovery of drugs targeting either liver or sexual stage parasites faces even greater gaps in knowledge. For example, the only drugs that target liver stage parasites are the long-ago-discovered 8-aminoquinolines (such as primaquine and tafenoquine, whose modes of action are unknown) or are the products of discovery efforts for asexual blood stage parasites (such as atovaquone, an inhibitor of the mitochondrial cytochrome *bc1* complex that is part of the electron transport chain in this organelle) (45, 46). Why are drugs with activity against asexual blood stage parasites so often ineffective against liver stage parasites, and which biochemical pathways constitute the best targets for developing drugs with activity against liver stage parasites? Recent advances in visualizing the sporozoite invasion process and the discovery of merosomes might soon reveal new targets for drug and vaccine discovery (47–49). However, only a few laboratories are able to produce liver stage forms of human parasites because of the technical restrictions of establishing the specialized insectariums required to produce infectious sporozoites in *Anopheles* mosquitoes and the difficulties of generating large numbers of sporozoite-infected hepatocytes either in vitro or in vivo. Progress in this important area has therefore been slow. New drugs are urgently needed to target *P. vivax* liver stage parasites, including the dormant hypnozoite forms that can cause relapses, yet no in vitro methods exist to guide the drug discovery and development processes. Only an adequate investment in basic biological investigations of the *Plasmodium* life cycle, focusing on *P. falciparum* and *P. vivax*, can provide the knowledge needed to identify new targets and strategies for prophylaxis or treatment.

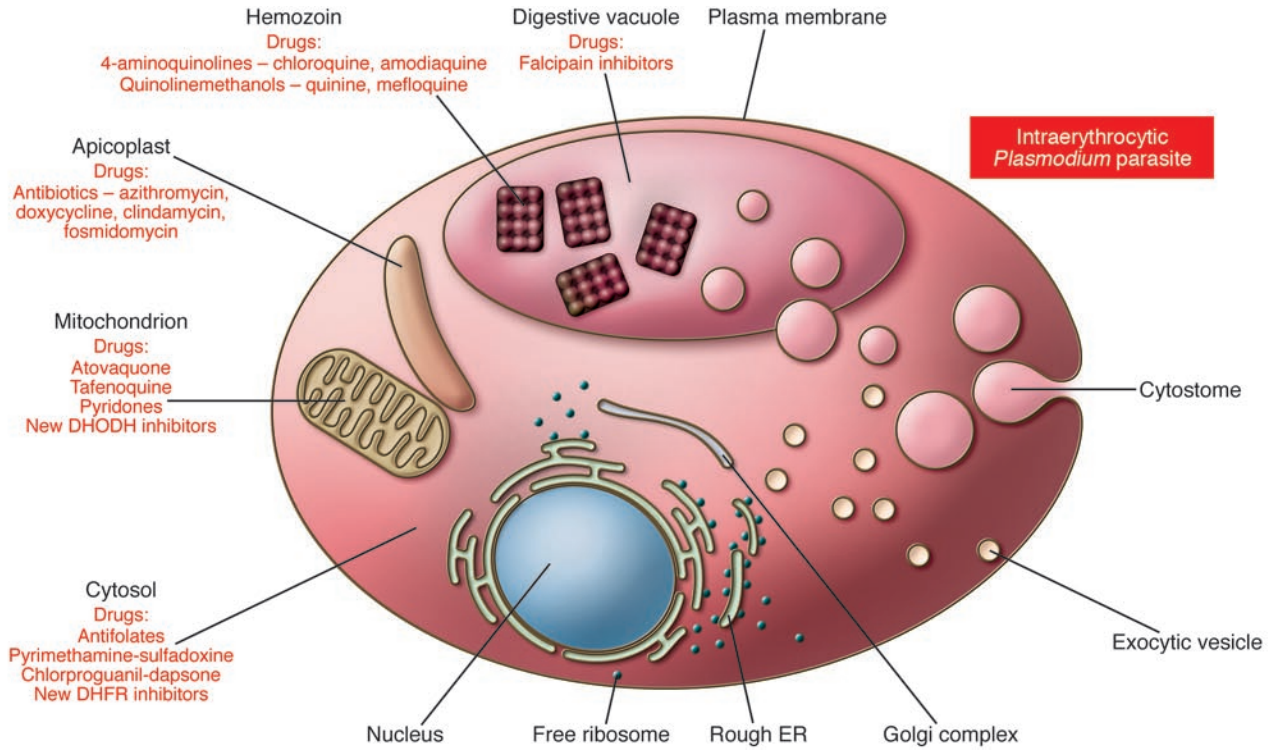

Figure 2
Antimalarial drugs mediate their effects by disrupting processes or metabolic pathways in different subcellular organelles. The 4-aminoquinolines, including chloroquine and amodiaquine, and the quinolinemethanols, including quinine and mefloquine, concentrate inside the acidic digestive vacuole, where they are believed to bind β-hematin and interfere with heme detoxification. The falcipain inhibitors that are under development target cysteine proteases that participate in hemoglobin degradation in this digestive vacuole. Antibiotics such as azithromycin, doxycycline, and clindamycin act inside the chloroplast-like plastid organelle, where they inhibit protein translation, resulting in the death of the progeny of drug-treated parasites (the "delayed-death" phenotype). Atovaquone and select other compounds inhibit electron transport in the mitochondrion, whereas antifolates disrupt de novo biosynthesis of folate in the cytosol. Only drugs for which the site of action is known with confidence are assigned to a subcellular location. Indeed, the targets and sites of action of other antimalarials, including artemisinin and artemisin derivatives, remain an area of active investigation. Reproduced with permission from Nature Publishing Group (44).

To adequately treat malaria, drugs must be fast acting, highly potent against asexual blood stage infections, minimally toxic, and affordable to residents of endemic regions. Drugs are also used to control malaria. For example, intermittent presumptive treatment (IPT) with sulfadoxine-pyrimethamine during second and third trimesters improves pregnancy outcomes (50) and is recommended as part of routine antenatal care throughout Africa. IPT strategies might also benefit infants (51, 52). The spread of *P. falciparum* resistant to the former first-line antimalarials chloroquine and sulfadoxine-pyrimethamine (53–55) has had a devastating impact on malaria treatment and control and has spurred multiple investigations into the development of new antimalarials, with an emphasis on artemisinin-based combination therapies (ACTs) (56, 57). ACTs combine a derivative of the natural product artemisinin, an extremely potent and fast-acting antimalarial endoperoxide, with a longer-lasting partner drug that continues to reduce the parasite biomass after the short-lived artemisinin has dropped below therapeutic levels. Artemisinin derivatives act rapidly against asexual blood stage parasites to alleviate symptoms and have the additional beneficial effect of killing gametocytes and therefore decreasing parasite transmission. Distinct modes of action of artemisinins and partner drugs should, in theory, enable the combination to kill parasites that manifest decreased susceptibility to one agent. Clinical studies in Thailand have shown excellent efficacy with the ACT mefloquine-artesunate, despite the relatively facile acquisition of parasite resistance to mefloquine (58). Mefloquine, however, is comparatively expensive and presents toxicity concerns. Current efforts are therefore focused on evaluating the impact of other ACTs, including artemether-lumefantrine, dihydroartemisinin-piperaquine, and artesunate-amodiaquine, when they are deployed as new first-line treatments. Most countries in the world have now switched to an official policy of using an ACT as the first-line treatment.

Will a worldwide switch to ACTs substantially and sustainably reduce the global burden of malaria? We would argue a qualified yes. The potency and clinical efficacy of ACTs, as well as the lack of proven cases of artemisinin treatment failure after ACT therapy until recently, attest to the promise of these drug combinations to dramatically reduce malaria. This occurred in KwaZulu-Natal where indoor residual spraying (IRS), active case detection, and deployment of artemether-lumefantrine in 2001 rapidly reversed an epidemic (9). Achieving similar results in other African nations, which unlike South Africa suffer from inadequate infrastructure and high transmission rates, is uncertain and will require sustained investments in health care delivery as well as subsidies for new drugs and other measures. Recent evidence that a similar impact can be achieved comes from a study in Zanzibar (8), which

reported a dramatic reduction in the rates of malaria-associated morbidity and mortality within two years of administering either artesunate-amodiaquine or artemether-lumefantrine free of charge to patients with malaria presenting at public health facilities (11). The subsequent combination of treatment with ACTs and distribution of insecticide-treated bednets (ITNs) produced a 10-fold reduction in the prevalence of parasitemia within a year.

Yet substantial concerns about ACTs remain. First, will repeated use of artemisinin derivatives cause toxicities similar to those observed in animal models (59, 60)? For example, brainstem neurotoxicity with chromatolysis has been observed in rats after high and repeated parenteral doses of artemisinins; and dihydroartemisinin-induced damage to primitive erythrocytes during yolk sac hematopoiesis as well as embryonic abnormalities and resorptions occurred in rats if the drug was administered during a short window of time after conception. This issue requires a detailed analysis, particularly in children and pregnant women — the individuals that get malaria and malaria treatments most frequently. Second, will resistance (inevitably) arise, and how can this be delayed? In vivo resistance to artemisinin and artemisinin derivatives has been selected in a *P. chabaudi* rodent malaria line that was repeatedly passaged in the presence of increasing concentrations of either artemisinin or artesunate (61). This result illustrates that resistance can occur in *Plasmodium* parasites and was obtained in a genetically tractable model that can be used to define resistance determinants.

Clinical data have revealed that ACT clinical failure can result from parasite resistance to the partner drug, and amplification of the *pfmdr1* gene has been identified as a key determinant of resistance to mefloquine and lumefantrine in Southeast Asian strains of *P. falciparum* (62, 63). This amplification is believed to result in overexpression of the PfMDR1 transporter, located on the membrane of the digestive vacuole within which hemoglobin degradation and heme detoxification occur. PfMDR1 overexpression might confer resistance by sequestering the drug away from its site of action. This mechanism does not seem to account generally for instances of resistance to mefloquine and lumefantrine in African strains of *P. falciparum* (64). Laboratory studies are urgently required to decipher determinants of resistance to ACT drugs in geographically distinct parasite strains. This should identify the drugs that are least prone to resistance and yield molecular markers that can serve as resistance sentinels. Mode-of-action studies are also essential to create a biological rationale for choosing new ACTs that have complementary and, ideally, synergistic activities and that will not readily fall prey to existing or newly acquired resistance mechanisms. In addition, pharmacokinetic/pharmacodynamic studies are required to optimize treatment regimes and doses and minimize recrudescence or selection for resistant parasites. Finally, discovery and development of new antimalarial drugs, separate from the artemisinins, must proceed so that replacements will be ready if and when ACTs reach the end of their clinical life.

Pathogenesis, immunity, and vaccines

Biology of pre-erythrocytic sporozoites and liver stage parasites. *Plasmodium* parasites encounter numerous anatomic barriers during their life cycle, and immune responses can interrupt parasite development either by blocking these key transitions or by directly killing the pathogen (Figure 1). Pre-erythrocytic sporozoites and liver stage parasites are very attractive targets for vaccines that aim to completely prevent infection (65–67). The advantage of targeting these forms of the parasite is that there are only small numbers to eliminate (68); at most, a few dozen sporozoites are transmitted during mosquito blood feeding and infect the liver. The pre-erythrocytic stages of the life cycle are completely asymptomatic and provide a relatively large window of opportunity for an effective immune response to eliminate the parasite (approximately 6 days for *P. falciparum*). Unfortunately, pre-erythrocytic infection in humans is experimentally intractable for practical purposes and consequently has remained poorly understood. In contrast, the pre-erythrocytic stages of disease in rodent models of malaria are amenable to direct experimental interrogation. Within the past few years, intravital imaging, gene knockouts, and other approaches have contributed to a cellular and molecular understanding of the initial stages of sporozoite transmission, liver infection, and liver stage development. Many of these findings have important implications for vaccine development.

When an infected *Anopheles* mosquito seeks a blood meal, it engages in repeated probing, each time releasing saliva and sporozoites into the dermal and subdermal tissue of the host (69). Sporozoites migrate through the skin to make contact with a blood vessel, then traverse the endothelium to enter the blood stream (49, 69). This skin phase of infection is highly susceptible to neutralization by antibodies that bind sporozoite surface proteins, mainly the circumsporozoite protein (CSP), effectively immobilizing the parasite (69). However, sporozoites sometimes gain immediate access to the blood stream, when mosquitoes directly cannulate a blood vessel. Sporozoites are then carried to the liver, entering the sinusoids through the portal fields. Sinusoids exhibit a highly specialized endothelium consisting mainly of fenestrated endothelial cells and stationary macrophages called Kupffer cells.

Sporozoites attach to the endothelial lining of liver sinusoids by interactions of their surface proteins (CSP and thrombospondin-related anonymous protein [TRAP]) with host extracellular matrix proteoglycans (70). CSP and TRAP are also crucial for sporozoite motility and infection of host cells (71). Sporozoites glide along the endothelium, then penetrate and traverse Kupffer cells to gain access to hepatocytes (72). Cell traversal seems to depend on at least two sporozoite secretory proteins, one of which contains a perforin-like membrane insertion domain that might allow the sporozoite to breach the cell membrane (73, 74). Importantly, sporozoites suppress the respiratory burst in Kupffer cells (75) and inhibit antigen presentation and cytokine release, which in turn might weaken an effective immune response against the parasite (76). Sporozoites can subsequently pass through a number of hepatocytes, which die by necrosis, before settling in a hepatocyte for further liver stage development (Figure 1) (77).

Liver stage development requires the formation of a specialized membrane compartment, called the parasitophorous vacuole (PV), in which the parasite is shielded from the host cell cytoplasm (78). Initial PV formation depends on secretory proteins that are characterized by 6-cysteine domains; sporozoites lacking 6-cysteine proteins enter hepatocytes but cannot form a PV (79) and do not undergo subsequent liver stage development. Additional proteins that the parasite inserts into the PV membrane might facilitate nutrient uptake from the host cell, as has been suggested for the UIS3 protein (80). Parasites lacking these PV proteins stop growing early in infection (81–83). 6-Cysteine protein–deficient (79) and PV protein–deficient parasites are thus effectively attenuated and cannot initiate blood stage infection (81–83).

Vaccines against pre-erythrocytic parasites. Parasites lacking either a 6-cysteine protein or a PV protein are live attenuated parasites that are powerful vaccines and build on groundbreaking work in the

1960s and 1970s that showed sterile, long-lasting protection in mice (84) and humans (85) after vaccination with radiation-attenuated sporozoites. In mice, single-dose immunization with specific genetically attenuated parasites can induce complete protection against subsequent sporozoite infection, and prime-boost immunizations induce protection for at least 6 months (83). Protection is, to some extent, mediated by antibodies that prevent sporozoite invasion of hepatocytes. $CD8^+$ T cells, however, are crucial and eliminate the remaining infected hepatocytes through both direct cytotoxicity and IFN-mediated mechanisms (83, 86, 87) similar to their role in protection induced by radiation-attenuated parasites (88). Efforts are underway to test *P. falciparum* parasites attenuated by deletion of the essential 6-cysteine proteins P52 and P36 (79), the essential PV membrane protein UIS3 (82), and the essential PV membrane protein UIS4 (81), as vaccines in humans (www.sbri.org). A separate effort seeks to develop the logistical means to deliver a radiation-attenuated vaccine (www.sanaria.com), and these delivery approaches could equally be applied to a genetically attenuated parasite.

Subunit vaccines have achieved lower efficacy than whole-parasite vaccines in phase IIa clinical trials (89, 90) but are logistically simpler. After a long history of disappointing results with other candidates, one subunit vaccine called RTS,S has shown promise in phase IIb clinical trials (91–94). RTS,S incorporates a fusion protein — which comprises CSP and the HBV surface antigen and aggregates as virus-like particles — and the adjuvant AS02, which is based on monophosphoryl lipid A and QS-21 (89). Vaccination with 3 doses of RTS,S prevented infection in 41% of malaria-naive adult volunteers in the US when they were challenged with sporozoites delivered by mosquito bite (90). In The Gambia, vaccination of adults naturally exposed to malaria-causing parasites with 3 doses of RTS,S achieved 74% efficacy in delaying the appearance of parasitemia during the first 9 weeks following vaccination, but this efficacy waned to 0% by the end of the transmission season; a booster dose the following season provided 47% efficacy during the first 9 weeks (91).

In Mozambique, 3 doses of RTS,S/AS02A given at monthly intervals to infants (93) and to children age 1–4 years (94) reduced the risk of infection with *P. falciparum*. Unlike in the studies in adults in The Gambia, the benefits in children seemed to be sustained for more than a year, with an overall vaccine efficacy of 35.3% against clinical malaria and 48.6% against severe malaria during 18.5 months of observation after the third dose of vaccine. These encouraging results have prompted plans for a large, multicenter trial that might lead to licensure of the product. The demonstration that the vaccine retains its efficacy when the AS02 adjuvant is lyophilized and reconstituted at the time of immunization, rather than coformulated at the time of manufacture, might simplify the logistics of delivering the vaccine (95). The results are also stimulating the search for additional pre-erythrocytic antigens that improve upon this success, either in combination with the CSP antigen or alone.

Immunity and vaccines against blood stage parasites. Vaccines against pre-erythrocytic stage parasites, such as RTS,S, have been designed to prevent infection and thereby prevent disease. Vaccines against the pathogenic asexual blood stages also have a strong rationale, but their primary goal is to prevent disease and not infection. Immunity to blood stage parasites is naturally acquired, limits parasitemia, and prevents disease; it is passively transferred to children in the IgG fraction of immune serum (96). Furthermore, immunity that prevents severe malaria is acquired rapidly, perhaps after only one or two episodes of severe disease (97), suggesting that the target antigens have limited diversity.

Liver stage development is a single cycle for the parasite, whereas asexual blood stage development is a repeating cycle, each cycle terminating with the release of a new brood of merozoites that invade fresh erythrocytes in only a few seconds. The ability of the merozoite to specifically attach to and invade erythrocytes is essential for blood stage development; for example, *P. vivax* must bind to the Duffy antigen to invade reticulocytes (98). This and other findings have inspired the search for merozoite antigens that elicit antibodies that block parasite invasion of erythrocytes. However, none of the merozoite antigens that have been tested in humans, including merozoite surface protein–1 and apical merozoite antigen–1, have yet been shown convincingly to confer high levels of protection. The Duffy antigen–binding protein of *P. vivax* is soon to be tested as a vaccine in humans, to determine whether antibodies blocking this essential receptor-ligand interaction can confer protection (99). Unlike *P. vivax*, *P. falciparum* uses multiple redundant pathways to invade erythrocytes, complicating the effort to develop anti-invasion vaccines against the latter (100).

Blood stage immunity might also target parasite proteins that are variably expressed on the surface of infected erythrocytes (IEs). These proteins are exported by intraerythrocytic parasites for specialized functions such as adhesion to endothelium and immunoevasion. The best example of this has been demonstrated during pregnancy. In women who are pregnant, *P. falciparum* parasites emerge that express distinct IE surface proteins, allowing these IEs to bind chondroitin sulfate A (CSA) and sequester in the placenta (101). First-time mothers lack antibodies specific for the IE surface proteins of these parasites and are highly susceptible to infection and disease (102). Women become resistant over successive pregnancies as they acquire antibodies that block IE binding to CSA (102). Placental parasites express distinct genes and proteins (103), including an IE variant surface protein called VAR2CSA (104) that is required for adhesion to CSA in some parasite lines (105) and that binds CSA in vitro (106). A program to develop a vaccine based on VAR2CSA or the other proteins expressed by placental parasites is well under way (107).

Preventing malaria in pregnant women offers a paradigm for vaccines that prevent specific syndromes by blocking sequestration of distinct parasite forms. An alternative vaccine model targets parasite toxins that can cause inflammatory responses and severe sequelae; that is, the vaccines target not the parasite causing the infection but the mediators of disease. The febrile paroxysms of malaria occur as merozoites are released from IEs, and the glycosylphosphatidylinositol (GPI) tail that is common to several merozoite surface proteins has been implicated as a key parasite toxin (108). Unlike host GPIs, parasite GPIs contain palmitic or myristic acids at C-2 of inositol and lack phosphoethanolamine substitution in core glycan structures (109). Vaccination with parasite GPI induces protection from disease in animal models (110), and antibodies specific for parasite GPI (mainly for the acylated phosphoinositol portion) are naturally acquired by humans in endemic areas; this is related to improved outcomes in some, but not all, studies (109, 111, 112).

Vaccination against sexual stage parasites. Vaccines against the sexual stages of the malaria parasite life cycle have been successful at preventing parasite transmission in experimental animals (113) and are being pursued as approaches to prevent transmission of both *P. falciparum* and *P. vivax*. Such vaccines will not provide any immediate direct benefit to the vaccinated individual, but their widespread deployment will help to reduce transmission of the para-

site and thus protect both the vaccinated individual and his/her community. Vaccines that block parasite transmission are likely to be used in combination with vaccines targeting other stages of the infection and might prevent the transmission of parasite escape mutants that arise to evade protective immune responses. When used in combination with vector control measures described below, vaccines that block transmission could play a key role in finally breaking the transmission of malaria-causing parasites, leading to eradication of the disease.

Vector biology and control

The intensity and pattern of transmission of malaria-causing parasites, and therefore the epidemiology of infection and disease, are largely a function of the seasonality, abundance, and feeding habits of the *Anopheles* mosquito vector. Where malaria elimination programs have been successful, such as those implemented in the US and Europe, vector control was an essential program component. Contemporary vector control strategies include ITNs, long-lasting ITNs (LLINs), and IRS. IRS with DDT was an essential component of the Global Malaria Eradication Programme in the past century and remains highly effective in regions where mosquitoes are sensitive to the insecticide, such as KwaZulu-Natal (9). ITNs have been shown to increase child survival substantially in studies at several sites across Africa (114–116). Current priorities for research on vectors include studies to sustain and/or enhance the effectiveness of existing methods, as well as efforts to develop novel strategies for vector-targeted malaria control.

Research to sustain current control methods. Resistance to pyrethroid insecticides is one of the most pressing research problems for vector biologists. Only pyrethroid insecticides are licensed for use in ITNs. Therefore, tools to rapidly detect the many genetic mechanisms that can underlie pyrethroid resistance are urgently needed. These insecticides act by binding to a voltage-gated sodium channel responsible for neuronal signal transmission. Unfortunately, pyrethroid resistance has appeared in many vector populations, particularly in Africa. Despite an insecticide program, vector populations rebounded in southern Mozambique when *Anopheles funestus* resistant to pyrethroid emerged in 1999 (117–119). More recently, pyrethroid resistance in *Anopheles gambiae* has been associated with program failure in Benin (120). Beyond these two clear examples, insecticide resistance has been detected in many other vector populations but not yet linked to any loss of effectiveness in malaria control programs (121, 122).

In this context, strategies are desperately needed to maximize the longevity of the pyrethroid insecticides used for ITNs and to develop and/or license new insecticides for malaria control. IRS strategies, although potentially more costly and difficult to implement than ITN and LLIN programs, have the advantage that they can be based on a broader group of licensed insecticides, including the well-known and cost-effective pesticide DDT. Unfortunately, DDT and pyrethroid insecticides target the same voltage-gated sodium channel protein. Furthermore, a set of mutations that alters protein structure and confers resistance to DDT also confers resistance to pyrethroids (123). Fortunately, not all forms of resistance to DDT and pyrethroid insecticides cross-react, and thus these two widely effective types of insecticides can often supplement or replace each other. However, the development of new insecticides with active compounds that have different target sites is a priority research area for malaria control.

Given the central place of DDT and pyrethroid insecticides in malaria control today, these two insecticides must be used judiciously. Because IRS programs put much larger amounts of insecticide into the environment than ITN programs, they effectively subject the vector population to higher levels of selection for resistance. For this reason, we believe that pyrethroids should not be used for IRS programs. Another insecticide, such as DDT or one that does not target the same voltage-gated sodium channel as DDT and pyrethroids, should be deployed for IRS programs, preserving pyrethroids for use in ITNs.

Development of new, vector-targeted malaria control strategies. New and improved strategies for malaria control are also motivating research on malaria vectors. One approach that offers the potential for near-term results is focused on the molecular and biochemical mechanisms that underlie key vector behaviors, for example, blood meal host selection (124, 125). Molecular pathways such as those involving the odorant receptors used in host finding and blood feeding are being studied as potential targets of novel, species-specific attractants and repellents. Another approach uses genetic manipulation to modify the ability of the natural vector population to transmit the pathogen. Exciting progress has been made in the investigation of genes encoding potential effector proteins that can interrupt parasite development in the mosquito (126, 127), as well as strategies to drive such genes into natural populations (128, 129).

Broad-based analyses of the genomes of vectors (130, 131) and pathogens, and the use of these new genomic data to better understand the complex population structure of natural vector populations, hold the key to long-term solutions. Ultimately, these kinds of research activities will not only advance new control ideas currently being contemplated but will also be the source of new approaches not yet recognized.

Conclusion

Over half a century ago, the development of chloroquine and DDT inspired an international campaign to eradicate malaria that made substantial progress in many areas, especially outside Africa. However, political and financial commitments waned, in parallel with the emergence and spread of chloroquine-resistant *Plasmodium* parasites and DDT-resistant *Anopheles* mosquitoes. A global resurgence of malaria ensued, including in areas where it had been largely eliminated.

Today, we are witnessing a redoubling of efforts and resources to attack the malaria problem, and this time the emphasis is on Africa, where the burden of malaria is greatest. As malaria comes under control in highly endemic areas, the pattern of infection and disease will change, with an increasing proportion of cases occurring in older children and adults and with an increased risk of local outbreaks. The latter will be especially likely to occur if control measures are allowed to lapse in the face of a decreasing burden of infection. Extensive surveillance will therefore be required to monitor these changes and to define optimal and cost-effective strategies for managing pre-elimination situation.

Meanwhile, resources for malaria research efforts remain meager, and the international community continues to face difficult decisions on how to balance efforts in discovery, development, and implementation of new tools. The emergence of artemisinin resistance is one of the greatest threats to renewed efforts to eradicate malaria, and reports from the Thai-Cambodian border are raising concerns that this might already be occurring (132).

Although current tools make it possible to quickly identify the genetic basis of drug resistance, do we have sufficient knowledge, tools, and multinational cooperation to effectively prevent the spread of resistant parasites?

Experience indicates that the most effective control programs are those that apply a combination of tools and that the efficacy of current interventions will one day be lost to a changing parasite or mosquito. Furthermore, our existing interventions are insufficient to meet the ambitious goal of global eradication. New concepts and tools are required to achieve eradication, hence the impetus to explore transmission-blocking and live attenuated parasite vaccines as well as anti-vector measures targeting novel processes. The *Plasmodium* parasite and its *Anopheles* vector offer many targets for intervention, and we have only just begun to harvest their genome sequences for insights into new interventions and a deeper understanding of host-parasite interactions. The future of malaria control and eradication efforts hinges on how well the scientific and public health communities can work together to extend the effective life span of our existing tools while discerning new interventions that interrupt the complex life cycle of *Plasmodium* parasites.

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Fetal Responses during Placental Malaria Modify the Risk of Low Birth Weight[▽]

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Inflammation during placental malaria (PM) is associated with low birth weight (LBW), especially during the first pregnancy, but the relative contribution of maternal or fetal factors that mediate this effect remains unclear and the role of gamma interferon (IFN-γ) has been controversial. We examined the relationship of maternal and cord plasma levels of IFN-γ, tumor necrosis factor alpha, interleukin-10, ferritin, and leptin to birth weight for Tanzanian women delivering in an area where there is a high rate of malaria transmission. The placental levels of inflammatory cytokines, including IFN-γ, increased significantly during PM in primigravid and multigravid women but not in secundigravid women. PM also increased maternal peripheral levels of all inflammatory markers except IFN-γ but had strikingly little effect on cord levels of these proteins. In a multivariate analysis, placental IFN-γ was negatively associated ($P = 0.01$) and cord ferritin was positively associated ($P < 0.0001$) with birth weight in infected (PM-positive [PM^+]) first-time mothers. This relationship was not observed in other mothers, consistent with the epidemiology of PM and disease. Cord leptin had a strong positive relationship with birth weight in offspring of PM-negative women ($P = 0.02$ to $P < 0.0001$) but not in offspring of PM^+ women (all differences were not significant) in the three gravidity groups. The results confirmed that placental IFN-γ is related to LBW due to PM during first pregnancies and suggest that fetal ferritin plays a protective role. Because fetal cells are a source of placental IFN-γ and cord ferritin, the fetal response to PM may modify the risk of LBW.

Placental malaria (PM) due to *Plasmodium falciparum* causes low birth weight (LBW), and this effect is estimated to kill tens of thousands or hundreds of thousands of infants annually (5, 13). PM is caused by parasite-infected erythrocytes that bind placental receptors, such as chondroitin sulfate A, to sequester in intervillous spaces (10). Women become resistant to PM over successive pregnancies as they acquire antibodies against placental or chondroitin sulfate A-binding parasite-infected erythrocytes (11, 35). First-time mothers lack these antibodies and commonly develop chronic PM with inflammatory infiltrates rich in monocytes and macrophages (19, 28). These inflammatory infiltrates have been associated with LBW and other poor pregnancy outcomes (16, 19, 34), but these relationships are seen primarily during first pregnancies.

Placental levels of the inflammatory cytokine tumor necrosis factor alpha (TNF-α) have also been related to LBW caused by PM (12, 33, 37), while the roles of gamma interferon (IFN-γ) and interleukin-10 (IL-10) have been controversial (12, 33, 37). TNF-α and IL-10 are expressed by the maternal inflammatory cells that infiltrate the infected placenta, while IFN-γ is secreted by placental villi, suggesting that the source is fetal (37). Elevated cord blood levels of ferritin, a marker of inflammation, were also recently found to increase the risk of LBW in a small study in Malawi (2). Other recent studies found elevated maternal levels of C-reactive protein (CRP) (3) and urokinase-type plasminogen activator (29) during infection, and the latter marker was significantly related to decreased birth weight. Taken together, these data suggest that inflammatory responses in the mother or fetus may play roles in the development of LBW, but the relative contributions of maternal or fetal factors remain unclear. No study of PM has analyzed all these potential risk factors in maternal and fetal blood simultaneously.

In healthy neonates, cord blood levels of the hormone leptin are positively associated with birth weight (39), but the role of leptin in PM has not been examined. Leptin, which regulates appetite and metabolism, is produced by adipocytes, and leptin levels normally reflect body fat stores (21). In addition to its role in metabolism, leptin potentiates inflammation by enhancing macrophage phagocytosis, as well as TNF-α and type 1 cytokine production by T cells (22, 30). In animal models, the leptin level increases during malaria infection (32). During pregnancy, the placenta acts as an additional source of leptin. Maternal plasma leptin concentrations increase (24) and do not correlate with body fat stores, indicating that leptin has an alternative function during pregnancy and fetal development (21). Fetal plasma leptin is derived from the placenta starting in early gestation (14) and from fetal adipose tissue which appears and develops progressively from 14 weeks of gestation to term (31).

Understanding the relative contributions of maternal and fetal factors that cause LBW during PM could focus the effort to develop interventions. In the present study, we measured levels of IFN-γ, TNF-α, IL-10, ferritin, and leptin in maternal and fetal blood samples from Tanzania. We examined whether

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TABLE 1. Characteristics of infants and mothers in the study population, stratified by maternal gravidity and PM status[a]

Group	PM status	*n*	Mean maternal age		Birth wt		Newborn gender	
			Age (yr)	*P* value	Wt (kg)[b]	*P* value	No. of males/no. of females	*P* value
Primigravidae	PM^-	185	20.8	0.07	3.101 (0.436)	0.0007	98/86[c]	0.3
	PM^+	47	19.8		2.851 (0.444)		21/26	
Secundigravidae	PM^-	153	23.3	0.25	3.242 (0.436)	0.001	82/71	0.1
	PM^+	33	24		2.987 (0.303)		14/19	
Multigravidae	PM^-	367	30.1	0.01	3.280 (0.397)	0.004	184/183	0.29
	PM^+	23	28		3.047 (0.468)		15/8	

[a] Maternal age and newborn birth weight data for the PM^+ and PM^- groups were compared by using an unpaired *t* test, and newborn gender data were compared by using a chi-square test. *P* values for the differences are indicated.
[b] The values are the means (standard deviations).
[c] The gender of the newborn of one PM^- primigravid mother was not recorded.

these levels were associated with LBW using multivariate analysis, and we assessed the effects of gravidity or PM on these relationships. Our results show that factors expressed by the fetus may modify the risk of LBW.

MATERIALS AND METHODS

Study subjects and clinical procedures. The subjects included in the present study were individuals participating in the Mother-Offspring Malaria Studies (MOMS) Project in Muheza district, northeastern Tanzania, between September 2002 and October 2005. Mother-infant pairs were recruited during delivery hospitalization at the Muheza Designated District Hospital. Clinical information, including antimalarial agent use during pregnancy to prevent malaria, was obtained by history and physical examination, as well as review of medical records. Pregnant women were eligible for the study if they were between 18 and 45 years old and had no clinical evidence of chronic or debilitating illness, such as recent significant weight loss or chronic diarrhea. Cases of twin or triplet gestation, human immunodeficiency virus infection, or early neonatal death were not included in the analyses in this study. LBW was defined as weight at birth of <2,500 g.

Protocols for procedures used in this study were approved by the International Clinical Studies Review Committee of the Division of Microbiology and Infectious Diseases at the U.S. National Institutes of Health. Ethical clearance was obtained from the institutional review boards of Seattle Biomedical Research Institute and the National Institute for Medical Research in Tanzania. Written informed consent was obtained from each mother before she entered the study.

Peripheral blood was obtained by venipuncture from women at delivery and anticoagulated with citrate phosphate dextrose. Cord blood samples were obtained by clamping the cord and cannulating umbilical vessels immediately after delivery. After removal of the umbilical cord and fetal membranes, placental blood samples were obtained by manual compression of the placental tissue in a grinder. Placental and cord blood samples were anticoagulated with EDTA. All samples were collected by trained nurses. Plasma was obtained by centrifugation at 3,000 × *g* for 3 min and was stored frozen at −70°C until it was thawed on the day that cytokine assays were performed.

Parasitemia was defined as identification of any parasites in a placental blood smear or a cord blood smear by microscopy. Thick and thin smears were prepared for all samples; thin smears were fixed with methanol. Blood slides were stained for 10 min in 10% Giemsa stain, washed in tap water, air dried, and then examined using light microscopy at a magnification of ×100. Ten thousand red blood cells were examined in the thin smear before it was concluded that a placental blood slide was negative.

Plasma analyte assays. Each plasma sample was analyzed using a multiplex, bead-based platform (BioPlex; Bio-Rad, Irvine, CA) and custom-made assay kits as previously described (8). For each serum sample, all analytes were assayed in a single day, thus eliminating freeze-thaw cycles. All pipetting and sample identification were performed with a bar code-enabled, high-speed pipetting robot (Megaflex; Tecan, Research Triangle Park, NC). The detection limits for the different soluble factors were as follows: IFN-γ, 0.04 pg/ml; TNF-α, 0.10 pg/ml; IL-10, 0.02 pg/ml; ferritin, 0.07 ng/ml; leptin, 1.28 pg/ml; and CRP, 0.01 μg/ml. The levels of soluble factors were adjusted to account for dilution in anticoagulant at the time of sample collection.

Statistical analyses. Analyses were performed using Statview 5.0.1 (SAS Institute, Cary, NC). Differences in the proportions of placental blood samples with detectable IFN-γ from PM-positive (PM^+) and PM-negative (PM^-) women were analyzed by a chi-square test. Differences between groups in the levels of soluble factors or parasite densities were analyzed by a Mann-Whitney test or Kruskal-Wallis test, according to the number of groups. An unpaired *t* test was used to test for differences in maternal age and infant birth weight between the PM^+ and PM^- groups. Factorial analysis of variance was used to identify interactions between PM or gravidity on the relationship between soluble factors and birth weight. Univariate linear regression and multivariate linear regression were used to analyze the relationship of cytokines and other soluble factors to birth weight. Levels of IFN-γ, TNF-α, IL-10, leptin, and ferritin, as well as parasite density, were log transformed to obtain normal distributions for regression analyses.

RESULTS

Demographic characteristics of the study population. After twins and triplets, cases of human immunodeficiency virus infection, and early neonatal deaths were excluded, 808 mother-infant pairs remained for analysis. Demographic characteristics of the cohort stratified by PM and gravidity are shown in Table 1. A total of 103 study participants had PM at delivery, while parasites were detected in cord blood smears of five participants. Primigravid and multigravid women with PM were significantly younger than their PM^- counterparts. PM was associated with lower birth weight in all gravidity groups ($P < 0.05$ for all groups), but the smallest newborns were delivered by first-time mothers. Mean birth weight did not vary significantly based on the mother's history of taking antimalarials as preventive treatment during pregnancy (data not shown), possibly due to the known high rates of drug-resistant parasites in the study area. According to the Dubowitz score, the gestational age of LBW newborns was not significantly different from that of other newborns (data not shown), suggesting that intrauterine growth retardation was the primary cause of LBW in this cohort. Infected primigravid women had significantly higher parasitemia levels (median percentage of red blood cells infected, 2.9%; $P = 0.0002$) than infected secundigravid women (median, 0.6%) or multigravid women (median, 0.9%).

PM alters maternal but not fetal levels of inflammatory markers. PM was associated with significant increases in the levels of inflammatory markers (including IFN-γ and TNF-α)

TABLE 2. Levels of soluble factors in cord, placental, and peripheral blood of primigravid women, secundigravid women, and multigravid women, stratified by PM status

Location	Cytokine	PM^- women		PM^+ women		*P* value[b]
		No. of samples	Median concn (interquartile range)[a]	No. of samples	Median concn (interquartile range)[a]	
Primigravid women						
Cord	TNF-α	168	105.4 (63.9–180.2)	40	118.2 (70.5–155.7)	NS
	IFN-γ	168	0.0 (0.0–0.0)	40	0.0 (0.0–0.0)	NS
	IL-10	168	3.5 (1.8–5.7)	40	3.3 (1.9–8.0)	NS
	Ferritin	168	101.3 (52.4–187.1)	40	140.2 (69.8–194.4)	NS
	Leptin	168	2,917.8 (1,091.5–6,811.4)	40	2,000.2 (754.4–6,193.7)	NS
Placenta	TNF-α	165	282.8 (161.9–535.1)	40	359.6 (287.7–668.6)	0.005
	IFN-γ	165	0.0 (0–75.5)	40	55.7 (0–190.9)	0.004
	IL-10	165	10.0 (6.8–17.5)	40	67.3 (29.2–180.0)	<0.0001
	Ferritin	165	776.6 (445.4–1,061.8)	40	1,024.0 (651.5–1,739.1)	0.0002
	Leptin	165	10,475.5 (4,774.0–20,984.2)	40	12,083.1 (7,732.8–28,661.3)	NS
Peripheral	TNF-α	165	24.1 (9.8–49.0)	41	70.6 (31.7–189.1)	<0.0001
	IFN-γ	165	0.0 (0.0–0.0)	41	0.0 (0.0–6.6)	NS
	IL-10	165	5.7 (3.5–11.4)	41	22.7 (13.8–64.8)	<0.0001
	Ferritin	165	14.5 (8.3–33.0)	41	74.6 (22.5–150.5)	<0.0001
	Leptin	165	2,448.7 (1,145.6–6,348.7)	41	940.8 (634.1–10,269.8)	0.0004
Secundigravid women						
Cord	TNF-α	141	118.8 (63.4–179.6)	31	101.5 (50.9–178.2)	NS
	IFN-γ	141	0.0 (0.0–0.0)	31	0.0 (0.0–0.0)	NS
	IL-10	141	2.3 (0.9–4.0)	31	3.5 (1.5–5.6)	0.03
	Ferritin	141	110.5 (68.4–171.9)	31	74.7 (44.3–153.5)	NS
	Leptin	141	2,568.9 (1,091.6–7,003.2)	31	3,441.6 (1,212.5–5,356.8)	NS
Placenta	TNF-α	135	324.1 (169.1–506.9)	31	263.2 (186.6–448.2)	NS
	IFN-γ	135	13.1 (0–68.1)	31	15.9 (0.-0–55.6)	NS
	IL-10	135	10.4 (5.6–17.5)	31	30.9 (14.5–57.3)	<0.0001
	Ferritin	135	639.5 (404.5–972.0)	31	613.4 (441.5–1,030.1)	NS
	Leptin	135	8,090.0 (4,279.9–16,420.0)	31	11,917.3 (3,475.7–21,359.0)	NS
Peripheral	TNF-α	134	21.8 (11.0–40.1)	31	55.0 (24.7–79.1)	0.0003
	IFN-γ	134	0.0 (0.0–0.0)	31	0.0 (0.0–0.0)	NS
	IL-10	134	6.1 (3.1–10.0)	31	22.6 (14.0–41.4)	<0.0001
	Ferritin	134	12.2 (7.4–20.5)	31	38.2 (21.2–79.9)	<0.0001
	Leptin	134	2,318.6 (1,111.0–6,581.3)	31	2,268.6 (446.8–4,679.7)	NS
Multigravid women						
Cord	TNF-α	340	103.1 (58.6–149.9)	23	149.3 (85.9–171.5)	NS
	IFN-γ	340	0.0 (0.0–0.0)	23	0.0 (0.0–0.0)	NS
	IL-10	340	2.8 (0.9–5.2)	23	2.7 (0.9–6.6)	NS
	Ferritin	340	90.2 (47.8–164.6)	23	105.7 (80.5–155.5)	NS
	Leptin	340	3,577.6 (1,725.6–6,880.1)	23	2,445.6 (881.8–3,892.9)	0.05
Placenta	TNF-α	328	263.6 (158.7–439.8)	20	512.3 (301.2–746.4)	0.005
	IFN-γ	328	0.0 (0–55.7)	20	49.3 (6.2–265.4)	0.001
	IL-10	328	9.8 (6.0–17.6)	20	49.8 (30.4–204.7)	<0.0001
	Ferritin	328	640.1 (385.8–960.6)	20	867.3 (746.4–1,196.4)	0.0007
	Leptin	328	7,193.0 (3,049.9–14,887.0)	20	9,495.4 (5,165.5–27,492.2)	NS
Peripheral	TNF-α	322	19.5 (9.0–44.4)	18	67.4 (17.9–98-6)	0.01
	IFN-γ	322	0.0 (0.0–0.0)	18	0.0 (0.0–0.0)	NS
	IL-10	322	6.2 (2.9–12.1)	18	27.2 (14.0–54.5)	<0.0001
	Ferritin	322	11.9 (6.9–28.0)	18	45.3 (12.0–97.9)	0.004
	Leptin	322	2,130.4 (895.9–5,236.7)	18	1,965.1 (1,108.0–5,776.6)	NS

[a] The concentrations of TNF-α, IFN-γ, IL-10, and leptin are expressed in picograms per milliliter, and the concentrations of ferritin are expressed in nanograms per milliliter.
[b] Differences between groups of women with and without PM were analyzed by using the Mann-Whitney U test. NS, not significant.

in the placental blood of primigravid and multigravid women but not in the placental blood of secundigravid women (Table 2). Placental IFN-γ was detected more frequently in samples from PM^+ primigravidae than in samples from PM^- primigravidae (68.2% versus 48.4%; $P = 0.02$) or multigravidae (84.2% versus 48.9%; $P = 0.003$) but not secundigravidae (56.7% versus 54.8%; $P > 0.999$). Similarly, the levels of ferritin, a marker of inflammation, as well as iron stores, were

significantly increased in placental blood of infected primigravid and multigravid women but not in placental blood of secundigravid women. The level of anti-inflammatory cytokine IL-10 was increased in placentas of infected women in all gravidity categories.

The levels of TNF-α, IL-10, and ferritin were also increased in the maternal peripheral blood of women with PM. Unlike the pattern seen in placental blood, the increases occurred in secundigravid women as well as in primigravid and multigravid women. The peripheral blood IFN-γ levels in PM^+ women were not significantly different from those in PM^- women.

The placental leptin levels in PM^+ samples were not different from those in PM^- samples for any of the gravidity groups. The peripheral leptin levels were significantly lower in PM^+ primigravid women than in PM^- primigravid women, but this relationship was not observed for other gravidity groups.

In general, the fetal levels of cytokines and other soluble factors were not altered during PM. PM was related to a small but significant increase in the level of cord blood IL-10 in secundigravid women. PM did not significantly alter cord blood levels of TNF-α, IFN-γ, leptin, or ferritin in any of the gravidity groups. The levels of the inflammatory marker CRP were elevated (>8.2 μg/ml) in only 17/457 (3.1%) cord blood samples tested, and elevated levels of CRP were seen in similar proportions of PM^+ cases (4/62, 6.4%) and PM^- cases (13/395, 3.3%) ($P = 0.22$).

Gravidity and PM modify relationships of soluble factors to LBW. We examined the relationship between all measured analytes and birth weight by using univariate linear regression. Placental IL-10 had a significant inverse relationship with birth weight ($\beta = -0.114$, $P = 0.002$), while cord leptin had a significant positive relationship with birth weight ($\beta = 0.277$, $P < 0.0001$). Cord ferritin, placental IFN-γ, and placental TNF-α were not significantly related to birth weight in these aggregate analyses.

PM and gravidity are known to influence relationships between inflammatory cytokines and birth weight in malaria-exposed individuals, and the relationship is limited to first pregnancies (12). We performed factorial analysis of variance to examine whether gravidity and PM modified relationships between soluble factors and birth weight in this cohort. Interaction terms involving gravidity and/or PM significantly or nearly significantly modified relationships between birth weight and several soluble factors, including cord ferritin (significance of term PM * parity, $P = 0.04$) and placental TNF-α (significance of term placental TNF-α * PM, $P = 0.07$). We therefore stratified our analyses by gravidity (Fig. 1) and by PM status (Fig. 2).

In analyses stratified by gravidity, LBW was related to significant or nearly significant reductions in levels of cord leptin and cord ferritin in all gravidity groups (Fig. 1). Although the placental levels of TNF-α, IFN-γ, and IL-10 were elevated in primigravid and multigravid women delivering LBW babies compared to women delivering normal-birth-weight babies (data not shown), the differences were not significant.

In analyses stratified by PM status, LBW was strongly related to inflammatory cytokines when the mother was PM^+. The placental levels of TNF-α, IFN-γ, and IL-10 were all significantly and substantially increased in PM^+ mothers delivering LBW babies compared to women delivering normal-birth-weight babies (Fig. 2). Conversely, the cord levels of both leptin and ferritin were significantly decreased in LBW babies compared with normal-birth-weight babies, and this was observed for both PM^- and PM^+ groups (data not shown).

Placental IFN-γ is specifically related to decreased birth weight. We performed multivariate regression analysis of the factors that were related to birth weight in the univariate analyses (Table 3). For multivariate analysis, we stratified by gravidity and PM status, since these variables interacted with several plasma factors and modified their relationship to birth weight. Because one-half of women had undetectable placental IFN-γ levels (which therefore could not be normalized by transformation), we analyzed the groups with detectable and undetectable IFN-γ levels separately.

In PM^+ primigravidae with detectable placental IFN-γ, cord blood ferritin was strongly related to increased birth weight ($\beta = 0.80$, $P < 0.0001$), while placental IFN-γ was related to decreased birth weight ($\beta = -0.39$, $P < 0.01$). These relationships were not seen in other PM^+ women (Table 3). In multivariate analysis that excluded IFN-γ data but included all PM^+ primigravidae, cord blood ferritin was still related to increased birth weight ($\beta = 0.41$, $P = 0.01$), while placental TNF-α was related to decreased birth weight ($\beta = -0.50$, $P = 0.03$). The effect of cord blood ferritin was different in different groups. When placental inflammatory cytokines were related to decreased birth weight (such as IFN-γ or TNF-α in PM^+ primigravidae or TNF-α in PM^- secundigravidae without detectable IFN-γ), ferritin had a positive effect on birth weight. Conversely, ferritin had an inverse relationship with birth weight in PM^- secundigravidae with detectable IFN-γ, in whom none of the cytokines had an effect on the outcome (Table 3).

In PM^- women, cord blood leptin levels were significantly associated with increased birth weight in all gravidity groups. Leptin levels were not related to birth weight in PM^+ women.

DISCUSSION

Malaria-related LBW is a major public health problem in tropical countries and is thought to kill tens of thousands or hundreds of thousands of infants each year. We report here a comprehensive assessment of cytokines and other soluble factors measured in maternal and fetal blood that may be involved in the pathogenesis of malaria-related LBW. Our results confirm the relationship between placental inflammation and LBW during PM of first-time mothers and not other mothers. Notably, only placental IFN-γ remained significantly associated with decreased birth weight in the multivariate analysis. The associations of placental IFN-γ and cord blood ferritin with LBW indicate that fetal responses may modify the risk of LBW.

Elevated placental levels of TNF-α during PM have been a consistent finding at different study sites (12, 33, 36), but the effect of PM on placental IFN-γ levels has been controversial (12, 33). Differences between studies may be related to the sensitivity of various assays used to measure IFN-γ or to biologic differences between study sites with different malaria transmission intensities. As observed in a previous study (12), the effect of PM that elicited inflammatory cytokines in the placenta was limited to primigravid and multigravid women and was not observed in secundigravid women in this cohort.

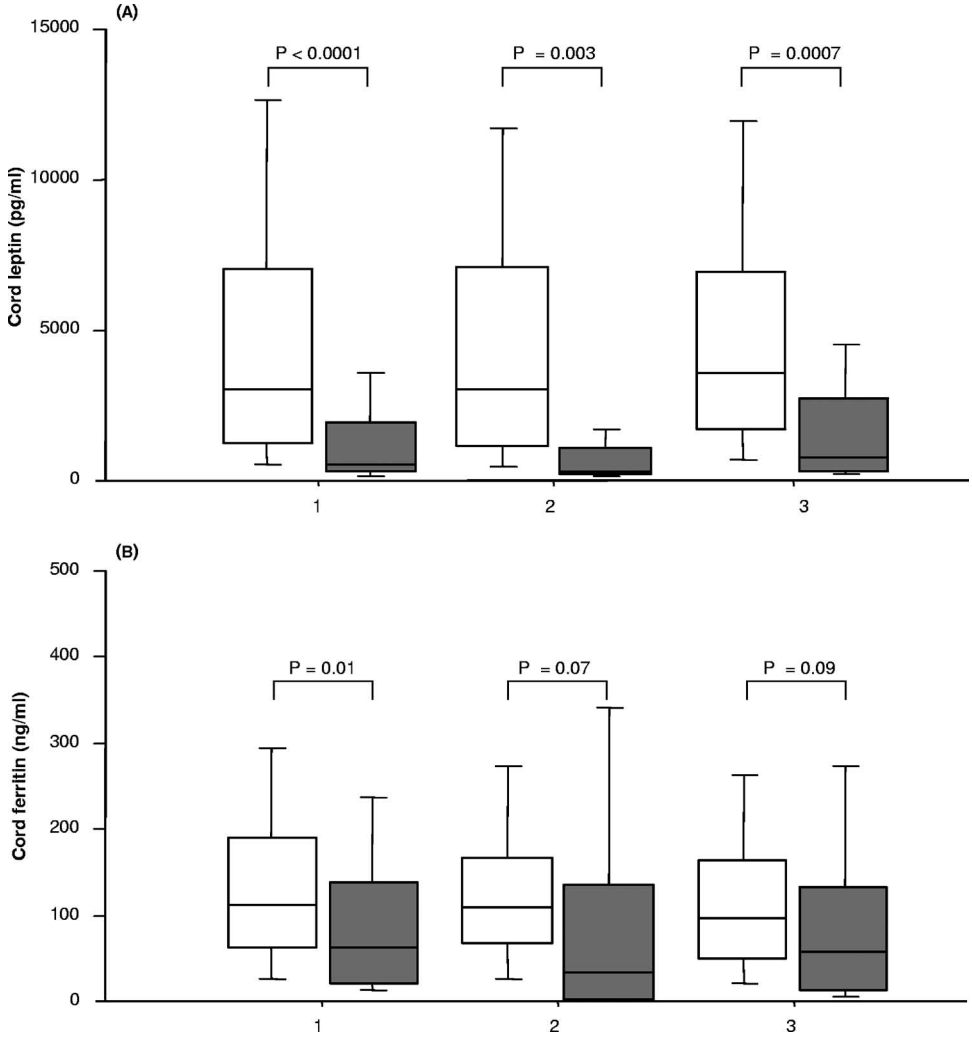

FIG. 1. Cord blood levels of (A) leptin and (B) ferritin for normal-birth-weight (open boxes) and LBW (shaded boxes) neonates. 1, primigravidae (188 normal-birth-weight babies and 20 LBW babies); 2, secundigravidae (170 normal-birth-weight babies and 5 LBW babies); 3, multigravidae (345 normal-birth-weight babies and 12 LBW babies). Each box plot indicates the median (horizontal line) and interquartile range (box), whereas the whiskers indicate the 10th and 90th percentiles. The differences between two groups were analyzed by the Mann-Whitney U test. Associated *P* values are shown.

The reasons that secundigravid women appear to be a distinct immunological group are unknown, but we speculate that secundigravidae comprise a transitional immune state between the full susceptibility of primigravidae and the significant immunity of multigravidae. This transitional state may involve distinct immune mediators in the response to infection, reflected by the distinct cytokine profile of secundigravidae.

Unlike the elevated levels measured in placental blood, the level of IFN-γ was not elevated in maternal peripheral blood during PM. This suggests that maternal or fetal cells in the placenta may be the primary source of IFN-γ during PM. In a previous study in Cameroon, IFN-γ was produced by fetal villi but not by maternal immune cells collected from infected placentas (37). The placenta secretes numerous cytokines and immunomodulatory molecules in addition to IFN-γ, and these molecules could play an important role in maternal and fetal outcomes. For example, a recent study in Tanzania found that fetal trophoblast cells express soluble vascular endothelial growth receptor 1 during PM, possibly as a strategy to modulate the maternal inflammatory response (26).

Our finding that PM did not substantially alter cytokines in fetal blood highlights the ability of the placental barrier to shield the fetus from inflammation. Proinflammatory cytokines like IL-1β, TNF-α, and IL-6 do not cross the placenta (1), which explains in part the unperturbed environment of the fetus. However, in utero sensitization to malaria antigens is not uncommon (9, 17, 23, 25, 38), indicating that the fetus is exposed to parasites or parasite antigens. The lack of fetal inflammation may suggest that fetal exposure to viable parasites does not occur or is limited or else that the fetus may actively suppress inflammation (for example, by increasing the number of regulatory T cells during PM) (6).

In the multivariate analysis of all the factors measured in the current study, only IFN-γ remained significantly associated with decreased birth weight, a relationship observed in PM^+ primigravidae but not in other groups. In areas where the rate

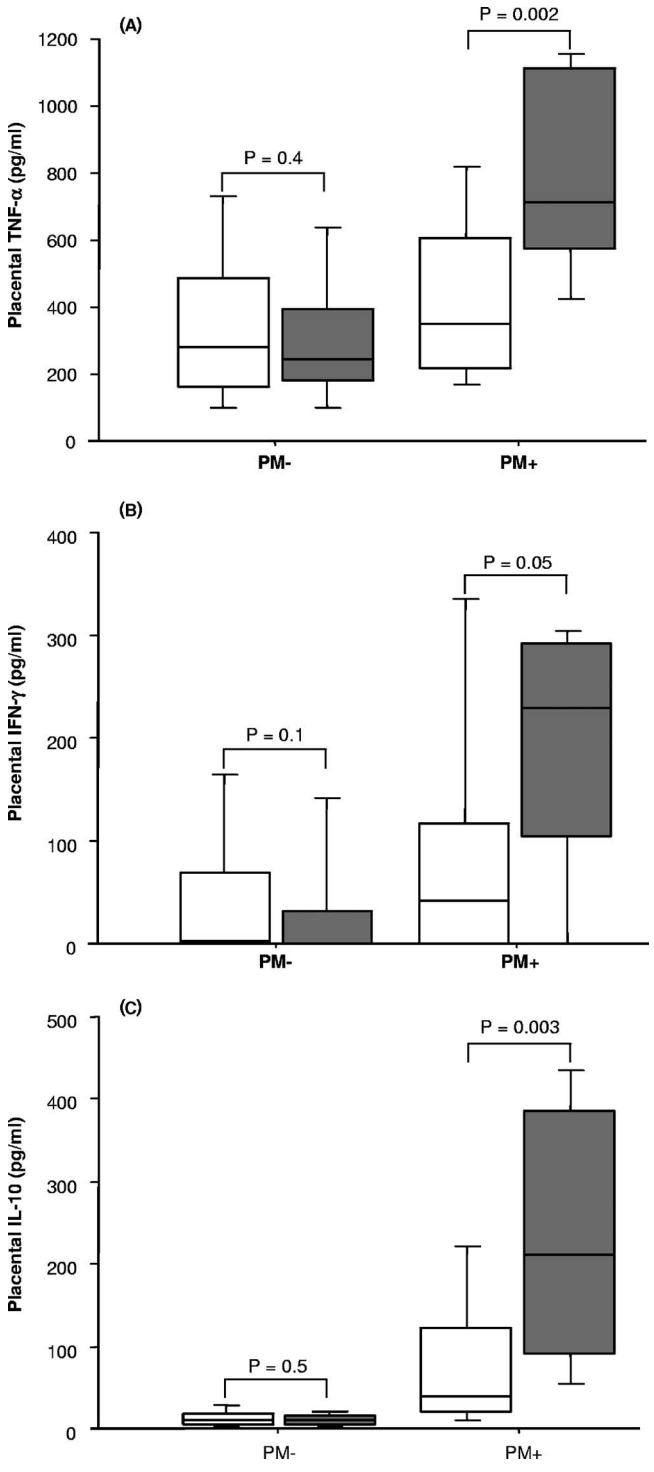

FIG. 2. Placental blood concentrations of (A) TNF-α, (B) IFN-γ, and (C) IL-10 for normal-birth-weight (open boxes) and LBW (shaded boxes) neonates. For the PM^+ group, there were 78 normal-birth-weight babies and 9 LBW babies; for the PM^- group, there were 603 normal-birth-weight babies and 26 LBW babies. Each box plot indicates the median (horizontal line) and interquartile range (box), whereas the whiskers indicate the 10th and 90th percentiles. The differences between two groups were analyzed by the Mann-Whitney U test. Associated *P* values are shown.

TABLE 3. Soluble factors related to birth weight in multivariate linear regression analysis stratified by PM and gravidity of women with detectable placental IFN-γ and without detectable placental IFN-γ

Women	*n*	PM status	Factor	β	*P* value[a]
Detectable placental IFN-γ					
Primigravid	80	PM^-	Cord leptin	0.393	<0.0001
			Cord ferritin	−0.079	NS
			Placental TNF-α	−0.283	NS
			Placental IL-10	0.111	NS
			Placental IFN-γ	−0.009	NS
Secundigravid	74	PM^-	Cord leptin	0.266	0.004
			Cord ferritin	−0.458	0.005
			Placental TNF-α	−0.261	NS
			Placental IL-10	−0.06	NS
			Placental IFN-γ	0.141	NS
Multigravid	161	PM^-	Cord leptin	0.191	0.007
			Cord ferritin	−0.117	NS
			Placental TNF-α	0.127	NS
			Placental IL-10	−0.101	NS
			Placental IFN-γ	−0.01	NS
Primigravid	27	PM^+	Cord leptin	0.079	NS
			Cord ferritin	0.8	<0.0001
			Placental TNF-α	0.065	NS
			Placental IL-10	0.208	NS
			Placental IFN-γ	−0.393	0.01
Secundigravid	18	PM^+	Cord leptin	0.252	NS
			Cord ferritin	−0.217	NS
			Placental TNF-α	0.393	NS
			Placental IL-10	−0.228	NS
			Placental IFN-γ	0.018	NS
Multigravid	16	PM^+	Cord leptin	0.053	NS
			Cord ferritin	0.168	NS
			Placental TNF-α	−0.591	NS
			Placental IL-10	−0.242	NS
			Placental IFN-γ	0.157	NS
No detectable placental IFN-γ[b]					
Primigravid	85	PM^-	Cord leptin	0.318	0.0002
			Cord ferritin	0.192	NS
			Placental TNF-α	−0.079	NS
			Placental IL-10	0.16	NS
Secundigravid	61	PM^-	Cord leptin	0.206	0.02
			Cord ferritin	0.475	0.0001
			Placental TNF-α	−0.597	0.009
			Placental IL-10	0.289	NS
Multigravid	167	PM^-	Cord leptin	0.277	<0.0001
			Cord ferritin	−0.08	NS
			Placental TNF-α	0.15	NS
			Placental IL-10	0.03	NS
Primigravid	13	PM^+	Cord leptin	−0.511	NS
			Cord ferritin	0.11	NS
			Placental TNF-α	−1.444	NS
			Placental IL-10	−0.09	NS
Secundigravid	13	PM^+	Cord leptin	0.277	NS
			Cord ferritin	−0.459	NS
			Placental TNF-α	0.169	NS
			Placental IL-10	0.009	NS

[a] NS, not significant.
[b] For multigravid PM^+ women with no detectable IFN-γ there were not enough cases to perform an analysis ($n = 4$).

of malaria transmission is high, like Muheza, where LBW due to PM is mainly a problem of first-time mothers, this primigravid-specific association is consistent with the epidemiology of poor outcomes. The relationship between inflammation and

LBW was also restricted to first-time mothers in an area of Kenya where the rate of transmission is high (12).

This is the first study to suggest that fetal ferritin levels may play a protective role in intrauterine growth during PM episodes. In a previous study in Malawi, increased cord blood ferritin levels were associated with lower birth weight during PM (2), but in that study there were important differences that may explain the discordant findings. The Malawi study involved a small cohort consisting of 32 PM^+ mother-newborn pairs, and the analyses were not stratified by parity. In an unstratified analysis of our Tanzanian data, we did not observe a relationship between cord blood ferritin and birth weight.

In the United States, giving iron supplements to pregnant women has been shown to increase birth weight without an effect on maternal hemoglobin or maternal iron stores (7), suggesting that an increased amount of iron transferred to the fetus may have direct benefits for weight. The level of cord blood ferritin may reflect a similar process in our cohort, indicating either increased maternal-fetal transfer or increased fetal iron stores with attendant benefits for birth weight. However, ferritin levels were related to improved outcomes only in the groups in which placental IFN-γ or TNF-α had a negative effect on birth weight, suggesting that ferritin may specifically counteract the deleterious effects of placental inflammation.

Hepatocytes and immune cells are known to be sources of plasma ferritin, and plasma ferritin levels increase during inflammation and decrease during iron deficiency. In our cohort, the cord levels of ferritin and the inflammatory marker CRP were not related, suggesting that changes in ferritin levels were not due to systemic inflammation of the fetus. Ferritin is also actively expressed by the placenta. Immunolocalization studies suggested that there is abundant ferritin production in the placental stroma but that ferritin is also detectable in the endothelium of fetal vessels within the placenta (4). The abundance and localization of ferritin in placental tissue during PM have not been studied previously but are of interest because of the association of cord ferritin levels with increased birth weight in this study.

Cord blood leptin was positively associated with birth weight in PM^- mothers but not in PM^+ mothers. During pregnancy, the human placenta (40) and other fetal tissues, including adipose, heart, and liver tissues (15), produce leptin. Cord blood leptin levels correlate with fetal size (20, 27), and both placental and fetal leptin levels are decreased in pregnancies complicated by fetal growth retardation (18, 39). However, PM appears to disrupt the relationship between cord leptin and birth weight. This may indicate that leptin plays additional roles during PM, and further study is warranted.

In summary, PM increases inflammatory cytokine levels in placental and maternal peripheral blood but not in fetal blood. Second-time mothers are distinct because their placental levels of inflammatory cytokines do not increase during PM. Inflammatory cytokines in the placenta are associated with poor outcomes during PM, and this relationship is limited to first pregnancies, consistent with the epidemiology of PM and disease. In first-time mothers, placental IFN-γ is associated with decreased birth weight, while cord ferritin is associated with increased birth weight, suggesting that fetal responses to infection may play a key role in the pathogenesis of malaria-related LBW. Future studies should localize and quantify IFN-γ and ferritin expression by cell subsets in the placenta and relate the measurements to outcomes, including birth weight during PM episodes.

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Decreased Susceptibility to *Plasmodium falciparum* Infection in Pregnant Women with Iron Deficiency

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(See the editorial commentary by Daily and Wylie, on pages 157–8.)

Iron plus folate supplementation increases mortality and morbidity among children in areas of malaria endemicity in Africa, but the effects of supplementation on pregnant women in malaria-endemic areas remain unclear. In northeastern Tanzania, where malaria and iron deficiency are common, we found that placental malaria was less prevalent (8.5% vs. 47.3% of women; $P < .0001$) and less severe (median parasite density, 4.2% vs. 6.3% of placental red blood cells; $P = .04$) among women with iron deficiency than among women with sufficient iron stores, especially during the first pregnancy. Multivariate analysis revealed that iron deficiency ($P < .0001$) and multigravidity ($P = .002$) significantly decreased the risk of placental malaria. Interventional trials of iron and folate supplementation during pregnancy in malaria-endemic regions in Africa are urgently needed to ascertain the benefits and risks of this intervention.

Malaria and iron deficiency are major contributors to severe anemia during pregnancy in malaria-endemic areas [1]. Anemia in pregnant women increases the risk of perinatal mortality and morbidity and is associated with increased risk of preterm delivery and low birth weight [2]. Low dietary intake of bioavailable iron has been identified as an important factor in the development of iron deficiency and anemia. Therefore, programs that provide iron supplements to pregnant women have been implemented worldwide.

The interaction between iron level, iron supplementation, and susceptibility to infection, including maternal malaria, remains a concern. In a retrospective study from Papua New Guinea, Oppenheimer et al. [3] reported that, among women treated with intravenous iron, the frequency of perinatal malaria increased among primiparous women but not among multiparous women. Use of oral iron supplementation by Gambian multigravidae did not increase the prevalence or the severity of peripheral or placental parasitemia during a randomized placebo-controlled study [4]. The risk of *Plasmodium falciparum* malaria did not increase during prospective follow-up among pregnant Thai women who were receiving hematinic treatment for anemia, although the risk of *Plasmodium vivax* malaria did increase [5]. These studies have not resolved whether antenatal iron supplementation increases the risk of malaria, and no studies have examined the impact of iron status on malaria risk during pregnancy.

Studies involving children have also yielded various findings but suggest that iron level and supplementation modify the risk for malaria. In Kenya, clinical malaria was significantly less frequent among iron-deficient children [6]. Iron supplementation did not increase the risk of malaria among children under intensive health surveillance in Kenya [7] but increased the prevalence of parasitemia and splenomegaly among infants from Papua New Guinea [8]. In the largest study to date, routine supplementation of Zanzibari children with iron and folate increased the risk of severe malaria and death [9]. Taken together, these findings suggest that routine supplementation with iron or iron plus folate increases morbidity and mortality among children in malaria-exposed populations [9].

In this study, we examined the relationship between maternal iron status and placental malaria risk in an area of intense malaria transmission. We assessed the prevalence and severity of placental malaria in women with and women without iron deficiency, defined on the basis of ferritin levels. We also assessed whether maternal gravidity status affects the relationship between iron status and the risk of placental malaria.

Subjects, materials, and methods. Subjects included in the present study participated in the Mother-Offspring Malaria Studies Project in the Muheza district of northeastern Tanzania from September 2002 through October 2005. Clinical procedures have been previously described [10]. In brief, mothers

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Table 1. Characteristics of the study population, by gravidity status.

Characteristic	Primigravidae (n = 120)	Secundigravidae (n = 112)	Multigravidae (n = 213)	P[a]
Age, mean ± SD, years	20.8 ± 3.5	23.3 ± 3.3	31.0 ± 5.2	<.0001
Placental malaria status				
Positive	25	16	14	.0006
Negative	95	96	199	
Use of intermittent presumptive therapy				
Yes	69	64	120	.9
No	51	48	93	
Use of insecticide-treated bed net[b]				
Yes	15	9	25	.2
No	66	82	136	
Iron deficient				
Yes	82	93	174	.008
No	38	19	39	

[a] Age data were compared by means of the Kruskal-Wallis test, and all other data were compared by means of the χ^2 test.

[b] Data were missing for 112 women (39 primigravidae, 21 secundigravidae, and 52 multigravidae).

were sequentially recruited from among those admitted to Muheza Designated District Hospital for delivery. After mothers provided written informed consent, peripheral blood and placental tissue samples were obtained from them. Placental blood was obtained by compression of placental tissue in a mechanical grinder.

Placental malaria was defined as the identification of any parasites in a placental-blood slide by microscopy. Thick and thin smears were prepared for all samples; thin smears were fixed with methanol. Blood slides were stained for 10 min in 10% Giemsa stain, washed in tap water, air dried, and examined using light microscopy (original magnification, ×100). Ten thousand red cells were examined in a thin smear before it was concluded that a placental-blood slide was negative for parasites.

Ferritin was assayed as part of a 12-plex sandwich-capture assay, and C-reactive protein was evaluated as part of a 3-plex competitive-binding assay, as described elsewhere [11]. Iron deficiency was defined as a ferritin concentration <30 ng/mL for subjects with a C-reactive protein level of ≤8.2 ng/mL (i.e., iron deficiency in the absence of inflammation) or as a ferritin concentration of <70 ng/mL for those with a C-reactive protein level of >8.2 ng/mL (i.e., iron deficiency in the presence of inflammation) [12].

Differences between proportions were compared using the χ^2 test. Placental parasite densities were compared using the Mann-Whitney test. Simple and multiple logistic regression models were used to test for associations between placental malaria and the specified variables. Analyses were performed using Statview, version 5.0.1 (SAS Institute).

Results. A total of 445 women (120 primigravidae, 112 secundigravidae, and 213 multigravidae) recruited while hospitalized for delivery had their iron status determined (table 1). Intermittent presumptive therapy with antimalarial drugs was used by 56.8% of women, and values did not differ significantly by gravidity status (57.5% of primigravidae, 53.3% of secundigravidae, and 56.3% of multigravidae; $P = .9$). Insecticide-treated bed nets were used during pregnancy by 49 (14.7%) of 333 women, and values did not differ significantly by gravidity status (18.5% of primigravidae, 9.8% of secundigravidae, and 15.2% of multigravidae; $P = .2$).

Fifty-five (12.4%) of 445 women had placental malaria. The percentage of women with placental malaria decreased as gravidity increased: placental malaria was detected in 17.2% of primigravidae, 12.5% of secundigravidae, and 6.1% of multigravidae ($\chi^2 = 14.9$; $P = .0006$). A total of 349 (78.4%) of 445 women were iron deficient, and iron deficiency was significantly less common among primigravidae (68.3%), compared with secundigravidae (83.0%) and multigravidae (81.7%) ($\chi^2 = 9.9$; $P = .008$). Mean hemoglobin levels (±SD) among mothers with iron deficiency (11.2 ± 4.1 g/dL) did not differ significantly from those among mothers without iron deficiency (11.4 ± 3.8 g/dL) overall ($P = .7$) and by gravidity status ($P > .5$ for all).

Women with iron deficiency were significantly less likely to have placental malaria at delivery. Twenty-nine (30.2%) of 96 women with adequate iron stores had placental malaria, compared with 26 (7.5%) of 349 mothers with iron deficiency ($P < .0001$). Placental malaria and iron deficiency are related to gravidity; thus, gravidity could confound the relationship between placental malaria and iron deficiency. We therefore examined the relationship between placental malaria and iron deficiency according to gravidity status. The prevalence of placental malaria among iron-deficient mothers was significantly lower than that among mothers without iron deficiency in the primigravid group (8.5% vs. 47.3%; $P < .0001$) and the secundi-

Table 2. Logistic regression analysis of factors potentially related to the risk of placental malaria.

Factor	Placental malaria status, no. of patients		OR (95% CI)	*P*	Adjusted OR (95% CI)	*P*
	Positive	Negative				
Seasonal transmission status						
Low	23	203	1.00		. . .	
High	32	187	1.51 (0.85–2.68)	.15	. . .	
Use of insecticide-treated bed net						
No	37	247	1.00		. . .	
Yes	5	44	0.76 (0.28–2.04)	.58	. . .	
Use of intermittent presumptive therapy						
No	28	164	1.00		. . .	
Yes	27	226	0.70 (0.40–1.23)	.21	. . .	
Gravidity status						
Primigravid	25	95	1.00			
Secundigravid	16	96	0.63 (0.32–1.26)	.19	0.82 (0.40–1.70)	.59
Multigravid	14	199	0.27 (0.27–0.54)	.002	0.32 (0.15–0.66)	.002
Iron deficient						
No	29	67	1.00	. . .	. . .	
Yes	26	323	0.19 (0.10–0.34)	<.0001	0.20 (0.11–0.36)	<.0001

NOTE. CI, confidence interval; OR, odds ratio.

gravid group (10.7% vs. 31.6%; $P = .01$) but not the multigravid group (5.1% vs. 12.8%; $P = .08$).

Iron deficiency was also associated with a decreased density of placental parasitemia in infected mothers. The median percentage of placental red blood cells containing parasites was significantly less for women with iron deficiency, compared with women with adequate iron stores (0.9% vs. 1.7%; $P = .03$). This relationship was observed in primigravidae (4.2% vs. 6.3%; $P = .04$) but not in secundigravidae (0.6% vs. 0.5%; $P = .9$) or multigravidae (1.2% vs. 0.5%; $P = .7$).

We performed logistic regression to analyze the relationship of placental malaria to iron deficiency or other factors. In simple regression analysis, iron deficiency and multigravidity significantly decreased the risk of placental malaria (table 2). Although the odds of placental malaria were lower if mothers used intermittent presumptive therapy or insecticide-treated bed nets or if they delivered during seasons of low malaria transmission, these effects were not significant in this cohort (table 2). In multiple regression analysis, iron deficiency and multigravidity were independently associated with a significantly decreased risk of placental malaria (table 2).

Discussion. We found that placental malaria was less frequent among pregnant Tanzanian women with iron deficiency than among those with normal iron status and that this effect was greatest during the first pregnancy. This is the first study to report that iron deficiency confers protection from placental malaria. Iron deficiency was previously reported to decrease the risk of malaria in children [6]. Our results are also consistent with those of earlier studies that linked iron supplementation to an increased risk of malaria during pregnancy, particularly among first-time mothers. The findings emphasize the need for large interventional trials to determine the risks and benefits of iron supplementation in different groups of women exposed to malaria.

Iron supplementation increased the risk of malaria among primigravid women in Papua New Guinea [3] but not among multigravid women in Papua New Guinea [3] or the Gambia [4]. Consistent with these observations, we found that the effect of iron deficiency on decreasing the risk of malaria was greatest among primigravidae. The differences between gravidity groups may be related to acquired immunity. In areas of stable malaria transmission, women acquire antibodies against placental parasites over successive pregnancies [13], and malaria is less frequent and less severe in multigravidae. In our Tanzanian cohort, the prevalence of placental malaria among primigravid women was nearly 3 times the prevalence among multigravid women, supporting the notion that women develop specific responses over successive pregnancies that confer resistance to malaria. Acquired immunity may limit the ability of iron supplementation to increase the risk of malaria in multigravidae. This phenomenon could lead to regional differences based on transmission intensity in the risk-benefit assessment of iron supplementation for pregnant women.

Acquired immunity or intensity of health surveillance could explain the inconsistent results obtained in earlier studies of iron supplementation in children. For example, iron supplementation increased the risk of malaria among children from Papua New Guinea [8] but not among those from Kenya, where surveillance was intense [7]. Among children in Zanzibar who were receiving iron and folic acid, the risk of clinical malaria was 16%

greater than the risk for children who were not (95% confidence interval, 2%–32%; $P = .02$), and the risk of cerebral malaria as a cause of death was increased (relative risk, 1.70; 95% confidence interval, 1.08–2.68; $P = .02$). However, these effects were not seen in a subset of children who were under intensive health surveillance [9]. These results suggest that iron and folic acid supplementation may have adverse or deadly consequences but that other factors may modify the risks of these consequences.

Different mechanisms have been proposed to explain how the risk of malaria might increase with iron supplementation and decrease with iron deficiency. Iron is an important nutrient for host requirements and for the metabolism of invading pathogens. Nutritional immunity involves iron-withholding defense systems, such as iron-binding proteins that cause hypoferremia, which reduces the amount of iron available for parasites and other organisms. Alternatively, iron inhibits the expression of inducible nitric oxide synthase (iNOS), which subsequently down-regulates the formation of nitric oxide in macrophages. Nitric oxide appears to be critical to macrophage defense against *P. falciparum* [14], and thus iron deficiency may amplify iNOS-mediated defenses against this pathogen. Finally, by enhancing erythropoiesis and production of reticulocytes, iron supplementation might increase susceptibility to parasite species such as *P. falciparum* that preferentially infect young red blood cells.

Hemoglobinopathies confer protection from malaria and could have confounding effects on our analysis. In the Gambia [15], iron supplementation reduced the maternal hemoglobin level and the infant birth weight among mothers with the HbAS genotype. Menendez et al. [15, p. 292] speculated that the unfavorable responses to iron supplementation in HbAS women resulted from increased susceptibility to malaria, although "malariological surveillance was not intense." HbAS occurs at a frequency of 15.9% among newborns at our study site (unpublished data), but mothers in our cohort were not routinely tested for sickle-cell trait. Future trials should assess the effects of iron supplementation separately in women with and women without hemoglobinopathies. Parasitic helminths, which cause iron deficiency and have been associated with either susceptibility or resistance to malaria in various studies [16], are a potential confounding factor that should also be examined in future studies.

Malaria and anemia during pregnancy are major contributors to maternal and newborn mortality, but interactions between these conditions complicate efforts to prevent them. Iron deficiency is a common cause of anemia but appears to confer resistance to maternal malaria. Because anemia during pregnancy is commonly due to malaria acquired in tropical regions, treatment with iron may carry unrecognized risks. Children and pregnant women have the highest risk of malaria and malarial anemia, and iron is often given to these groups either as a supplement or as presumptive treatment for anemia. Recent trials have highlighted mortality risks among children in areas of malaria endemicity in Africa who received iron and folate supplementation. Similar randomized trials involving pregnant women are warranted in order to guide the optimal use of iron supplementation or treatment in this vulnerable group.

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Maternal peripheral blood level of IL-10 as a marker for inflammatory placental malaria

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Abstract

Background: Placental malaria (PM) is an important cause of maternal and foetal mortality in tropical areas, and severe sequelae and mortality are related to inflammation in the placenta. Diagnosis is difficult because PM is often asymptomatic, peripheral blood smear examination detects parasitemia as few as half of PM cases, and no peripheral markers have been validated for placental inflammation.

Methods: In a cohort of Tanzanian parturients, PM was determined by placental blood smears and placental inflammation was assessed by histology and TNF mRNA levels. Maternal peripheral blood levels of several immune mediators previously implicated in PM pathogenesis, as well as ferritin and leptin were measured. The relationship between the levels of these soluble factors to PM and placental inflammation was examined.

Results: Peripheral levels of TNF, TNF-RI, TNF-RII, IL-1, IL-10, and ferritin were elevated during PM, whereas levels of IFN-γ, IL-4, IL-5 and IL-6 were unchanged and levels of leptin were decreased. In receiver operating characteristic curve analysis, IL-10 had the greatest area under the curve, and would provide a sensitivity of 60% with a false positive rate of 10%. At a cut off level of 15 pg/mL, IL-10 would detect PM with a sensitivity of 79.5% and a specificity of 84.3%. IL-10 levels correlated with placental inflammatory cells and placental TNF mRNA levels in first time mothers.

Conclusion: These data suggest that IL-10 may have utility as a biomarker for inflammatory PM in research studies, but that additional biomarkers may be required to improve clinical diagnosis and management of malaria during pregnancy.

Background

Placental malaria (PM) due to *Plasmodium falciparum* is a major cause of mortality for mothers and their offspring, and is most frequent and severe during first pregnancies [1]. PM is caused by parasite-infected erythrocytes that bind to chondroitin sulfate A (CSA) and sequester in the

placenta [2]. In histologic studies, PM can appear as an acute condition with little to no inflammation, or as a chronic disorder with sometimes heavy inflammation and deposition of parasite haemozoin (also called pigment) [3]. Chronic inflammatory PM has been most closely related to poor maternal and foetal outcomes in earlier studies [4]. In areas of stable malaria transmission, first time mothers often develop chronic PM, with inflammatory infiltrates and elevated Type 1 cytokines in the placenta [4,5].

Antenatal diagnosis of PM by Giemsa-stained blood smears fails to identify a substantial proportion of PM cases [6], possibly as many as half [1] and no tools exist that can predict poor pregnancy outcomes. PCR-based detection of *P. falciparum* DNA in peripheral blood is frequently positive when peripheral blood smear is negative. However, PCR can detect dead parasites, free parasite DNA, or DNA in phagocytic cells, and PCR-detection is not associated with pregnancy outcomes [6]. Antigen capture tests show promise, but they yield information only on parasitaemia and not inflammation [7]. A recent study from Kenya reported an association between plasma urokinase receptor levels measured at delivery and low birth weight in maternal malaria [8], suggesting that host biomarkers may be useful for discriminating women likely to experience poor outcomes from other women. Peripheral biomarkers of placental inflammation may be of particular value, since this condition is related to poor outcomes. In the present study peripheral blood levels of several immune mediators and other proteins in a cohort of Tanzanian women was examined at the time of delivery, and their associations with PM and placental inflammation was determined.

Methods

Clinical procedures

Placental samples, peripheral blood and clinical information were provided by Tanzanian women aged 18 to 45 years delivering at the Muheza Designated District Hospital, Muheza, Tanga region, in an area of intense malaria transmission. These women were participating in a birth cohort study known locally as the Mother-Offspring Malaria Studies (MOMS) Project. Women signed an informed consent form before joining the study, and women with known HIV or HIV-related sequelae in their offspring were excluded. Routine microbiological testing for other infectious diseases was not performed at the study site. Clinical information was collected by project nurses and assistant medical officers on standardized forms. Study procedures involving human subjects were approved by the International Clinical Studies Review Committee of the Division of Microbiology and Infectious Diseases at the US National Institutes of Health, and ethical clearance was obtained from the Institutional Review Boards of Seattle Biomedical Research Institute and the National Institute for Medical Research in Tanzania.

Peripheral blood was collected in citrate phosphate dextrose around the time of delivery, and plasma was separated and frozen at -80°C. The placenta was collected at delivery, and a full thickness biopsy from the middle third of the placental disc was taken. Tissue was fresh frozen in liquid nitrogen and stored at -80°C. Placental blood samples were obtained by manual compression of the placental tissue in a grinder. Placental parasitaemia was defined as the identification of any parasites in a placental blood slide by microscopy. Thick and thin smears were prepared; thin smears were fixed with methanol. Blood slides were stained for 10 minutes in 10% Giemsa, washed in tap water, air-dried, then examined using light microscopy at 1000 × magnification. Ten thousand red cells were examined in the thin smear before concluding that a placental blood slide was negative.

Laboratory procedures

Plasma levels of cytokines, cytokine receptors, ferritin and leptin were analyzed using a multiplexed, bead-based platform (BioPlex®, BioRad, Irvine, CA) and custom-made assay kits as previously described [9]. Detection limits for these assays were as follows: TNF 0.10 pg/ml, TNF receptor (R) I 1.58 pg/ml, TNF-RII 0.21 pg/ml, IFN-γ 0.04 pg/ml, IL-1 0.01 pg/ml, IL-4 0.30 pg/ml, IL-5 0.02 pg/ml, IL-6 0.45 pg/ml, IL-10 0.02 pg/ml, ferritin 0.07 ng/ml, and leptin 1.28 pg/ml. Levels of soluble factors were adjusted to account for dilution in anticoagulant at the time of sample collection. For each plasma sample, all analytes were assayed in a single day, thus eliminating freeze/thaw cycles.

For histologic analysis, PM-positive tissue was selected and 5 mm cryosections of placental tissue were fixed in methanol and stained with Giemsa. Sections were assessed by examining greater than ninety 600 × fields per section. Immune infiltrates within the intervillous spaces were qualitatively scored as (-) for none or very few inflammatory cells present, (+) for inflammatory cells present. Histological analysis was performed by a single observer (A.M.).

Quantitative PCR was performed as described elsewhere [10]. Briefly total RNA was extracted from frozen cryosections using RNeasy minikits (Qiagen) and cDNA was synthesized using Superscript III enzyme (Invitrogen) and anchored oligodT20 primers. Real-time PCR was performed in duplicate using SYBR green master mix and an ABI Prism 7000 or 7500 (Applied Biosystems). Threshold cycles (CT) were calculated and normalized to CT of KRT7 (a gene expressed by trophoblasts and not by inflamma-

tory cells). Data are presented as fold-difference from control gene, calculated by $2^{(\text{control CT-gene CT})}$. The oligonucleotide primers used for PCR reactions included: TNF Forward CACGCTCTTCTGCCTGCT; TNF-α Reverse CAGCTTGAGGGTTTGCTACA; KRT7 forward: GGCTGAGATCGACAACATCA; KRT7 reverse: CTTGGCACGAGCATCCTT.

Statistical analysis

Student's t-test was used for the analysis of maternal age and birth weight within primigravid (first pregnancy) and multigravid (second and later pregnancy) groups. Mann-Whitney test was used to examine cytokine levels. Linear regression coefficients were calculated using simple regression analysis. Receiver operating characteristic (ROC) curve and area under the curve (AUC) analyses were performed with IL-10 and other soluble factors levels as continuous variables using JROCFIT and JLABROC4 algorithms that are available online at the URL [11]. Sensitivities and specificities of elevated IL-10 to detect PM were calculated at specific cutoff levels of 10 pg/ml, 15 pg/ml or 35 pg/ml. Other analyses were performed using Statview 5.0.1 (SAS Institute, Cary, North Carolina, United States).

Results

Peripheral plasma samples used for these studies were provided by 660 women delivering singleton live-born babies in Muheza, Tanzania. Clinical data are shown in Table 1. PM+ multigravid women were younger than PM- multigravid women, and birthweight was significantly lower in PM+ deliveries compared to PM- deliveries in both gravidity groups.

Peripheral levels of cytokines, leptin and ferritin vary during PM

Comparison of concentrations of cytokines and other soluble factors in maternal peripheral blood stratified for PM and parity is shown in Table 2. PM significantly increased peripheral levels of TNF, TNF-RII, IL-10 and ferritin in women of both parities. Peripheral levels of TNF-RI and IL-1 significantly increased while levels of leptin significantly decreased in primigravid but not multigravid women during PM. The levels of other soluble factors were similar between PM- and PM+ women.

Peripheral IL-10 levels are markers of PM and placental inflammation

The soluble factors that were significantly elevated in peripheral blood during PM were analyzed by ROC curve analysis to determine their utility as biomarkers to detect PM (Table 3). IL-10 had the greatest area under the curve (AUC) at 0.83 in first time mothers and 0.82 for all mothers, indicating the highest sensitivity and specificity. The ROC curve for IL-10 in first time mothers is shown in Figure 1. Using an IL-10 cutoff for a false positive rate of 10% would yield a sensitivity of 60%, whereas a cut off for sensitivity of 90% would yield a false positive rate of 50%. Ferritin and TNF-RII had AUC values greater than 0.75 in first time mothers. Derived values, resulting from the combination by summation or addition of IL-10 with either ferritin or TNF-RII provided no improvement in sensitivity and specificity (data not shown).

The ability of IL-10 elevations above various threshold values to discriminate infected from uninfected women was examined (Table 4). An IL-10 cutoff level of 15 pg/mL yielded values above 75% for both parameters. Peripheral IL-10 levels were specifically elevated in first time mothers who had placental inflammation by histology (Figure 2). Further, peripheral IL-10 levels correlated significantly with placental TNF mRNA (Figure 3).

Discussion

Peripheral blood smear analysis has low sensitivity to detect PM. PCR based and antigen capture tests for the diagnosis of PM have increased sensitivity but cannot detect inflammation, which is related to poor pregnancy outcomes. This study suggests that peripheral IL-10 levels may be a useful tool to identify women with inflammatory PM and therefore those likely to have poor pregnancy outcomes. Using a cut-off level of 15 pg/mL, IL-10 levels would detect PM with a sensitivity of 79.5% and specificity of 84.3%. IL-10 may have utility in longitudinal studies, examining the burden of malaria over gestation, when the placenta is not available for microscopic analysis. Future studies should measure IL-10 levels throughout gestation to assess relationships to antenatal parasitemia and to pregnancy outcomes.

Table 1: Characteristics of the study population. *

	Primigravidae			Multigravidae		
Characteristic	**PM- (n = 166)**	**PM+ (n = 39)**	**P**	**PM- (n = 415)**	**PM+ (n = 40)**	**P**
Maternal age in years (Mean; SD)	20.6 (3.3)	19.7 (1.9)	0.0964	28.9 (5.9)	25.7 (4.4)	**0.0008**
Birth weight in kg (Mean; SD)	3.10 (0.43)	2.83 (0.42)	**0.001**	3.25 (0.386)	3.04 (0.36)	**0.0014**

*Data presented are Mean (SD).

Table 2: Peripheral levels of cytokines and other soluble factors stratified by parity and PM status.*

	Primigravidae			Multigravidae		
Factor	**PM- (n = 166)**	**PM+ (n = 39)**	**P**	**PM- (n = 415)**	**PM+ (n = 40)**	**P**
TNF	22.9 [8.3–46.7]	62.1 [26.3–127.5]	<0.0001	18.7 [8.70–37.9]	57.7 [23.8–84.7]	0.0002
TNF- RI	948 [550–1411]	1374 [851–2290]	0.0003	812 [481–1249]	1004 [517–1580]	0.0978
TNF- RII	186 [0–494]	673 [260–1425]	<0.0001	190 [0–403]	590 [129–959]	<0.0001
IL-1	2.28 [0.72–4.36]	5.05 [1.70–11.3]	0.0018	2.06 [0.73–4.46]	3.06 [0.32–7.36]	0.1409
IL-4	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.7676	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.9054
IL-5	1.96 [0.39–4.21]	1.76 [0.12–3.32]	0.4215	2.09 [0.39–4.09]	1.90 [0.89–5.89]	0.3158
IL-6	18.4 [9.26–37.6]	29.3 [15.0–42.5]	0.083	13.1 [3.75–29.5]	13.2 [8.83–19.2]	0.7618
IL-10	5.69 [3.28–11.3]	23.4 [15.1–62.7]	<0.0001	6.12 [2.91–11.6]	22.2 [13.9–40.3]	<0.0001
IFN-γ	0.0 [0.0-0.0]	0.0 [0.0–3.67]	0.5083	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.6607
Leptin	2404 [973–6218]	1029 [658–4115]	0.0245	2161 [1014–5293]	1490 [452–5052]	0.3444
Ferritin	14.0 [8.00–32.4]	62.7 [20.8–144.8]	<0.0001	11.6 [6.7–25.9]	40.4 [19.3–82.0]	<0.0001

*Data are presented as median [interquartile ranges]. P-values were calculated by Mann-Whitney test.

IL-10 is a key cytokine both in protection and immunopathology during malaria. High levels of IL-10 observed during malarial episodes may be beneficial by reducing the inflammatory response, but may be detrimental by decreasing antiparasitic cellular immune responses. IL-10 is an anti-inflammatory cytokine that acts in part by blocking monocyte/macrophage production of inflammatory cytokines such as IL-6, TNF, and IL-l [12]. Animal studies have suggested that IL-10 may play a regulatory role during parasitic infection that modulates susceptibility. In particular, IL-10 inhibits the microbicidal activity of IFN-γ-treated macrophages against intracellular parasites such as *Toxoplasma gondii* [13], *Trypanosoma cruzi* [14] and *Leishmania major* [15] and the killing of extracellular *Schistosoma mansoni* schistosomulas [16]. These effects may result from decreased production of the toxic nitrogen oxide metabolites[17].

The blood stages of *P. falciparum* are also cleared by phagocytosis and killed by oxidative products of nitric oxide released by macrophages [18]. IL-10 has been previously observed to be elevated during malarial episodes in non-pregnant [19,20] and pregnant individuals [21]. Both increased and decreased levels of IL-10 have been associated with poor malaria outcomes. Low levels of IL-10 or low IL-10 to TNF ratios were associated with severe malarial anemia in African children [22,23] while high IL -10 levels were associated with reduced ability to eliminate malaria parasitaemia in Tanzanian children [24].

PM results from the accumulation of parasites that bind to CSA in the intervillous spaces of the placenta [2,25]. In response to the sequestered mass of parasites, inflammatory cells infiltrate the intervillous spaces This inflammatory infiltrate can be massive, and prominently features monocytes/macrophages. In vitro data suggests these cells

Table 3: Area under the Receiver Operator Characteristic (ROC) curve to detect PM.*

Soluble factor	All gravidities	Primigravidae
TNF	0.690	0.731
TNF- RI	0.635	0.694
TNF- RII	0.731	0.752
IL-1	0.608	0.658
IL-10	0.815	0.830
Ferritin	0.733	0.759

*Only cytokines and other soluble factors significantly elevated during PM are shown.

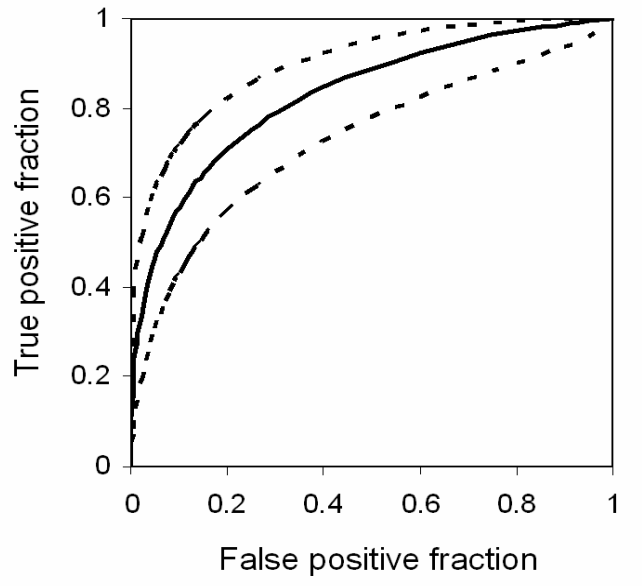

Figure 1
Receiver operator curve for peripheral IL-10 levels in first time mothers to detect PM. Solid line is the best fit curve; dashed lines show the 95% confidence intervals.

Table 4: Sensitivity and specificity of discrete IL-10 cut-off levels toclassify cases of PM in first time mothers (n = 205).

IL -10 levels	Sensitivity (%)	Specificity (%)
≥ 10 pg/mL	84.6	72.9
≥ 15 pg/mL	79.5	84.3
≥ 35 pg/mL	43.6	95.8

are the principal source of IL-10 [21]. In Kenyan children, high levels of peripheral blood IL-10 were positively correlated with binding of infected red blood cells to CD36 [26], but the relevance of this observation to malaria pathogenesis is unknown, and we find that levels of IL-10 also increase when CSA-binding parasites are the major parasite form causing infection. Placental levels of TNF increase during PM [5,21,27] and TNF gene expression is specifically related to placental inflammation [10]. Increased placental blood levels of TNF are related to poor outcomes for both the mother and her newborn [5,27]. In the present study, placental TNF mRNA positively correlated to peripheral blood IL-10 levels in first-time mothers, strengthening the association between peripheral IL-10 levels and placental inflammation.

The present data indicate that peripheral ferritin levels are also elevated during PM. Ferritin is a positive acute phase protein and is known to increase during infection and injury. In non-pregnant individuals, ferritin levels increase during both asymptomatic and symptomatic malaria, and the highest levels have been recorded in individuals with severe disease [28]. Serum ferritin may also increase in the presence of subclinical infection [29]. During the acute phase response, inflammatory cytokines such as IL-1β increase the synthesis of both heavy and light subunits of ferritin [30]. In this Tanzanian cohort, PM was associated with elevated levels of IL-1 and TNF in maternal peripheral blood, particularly among first time mothers who are most likely to experience placental inflammation. Ferritin is widely used for determining iron deficiency anemia in industrialized countries, and therefore has the advantage of existing diagnostic platforms. For this reason, ferritin should also be evaluated in prospective studies as a cost-effective antenatal assay for screening inflammatory PM and poor pregnancy outcomes in tropical countries.

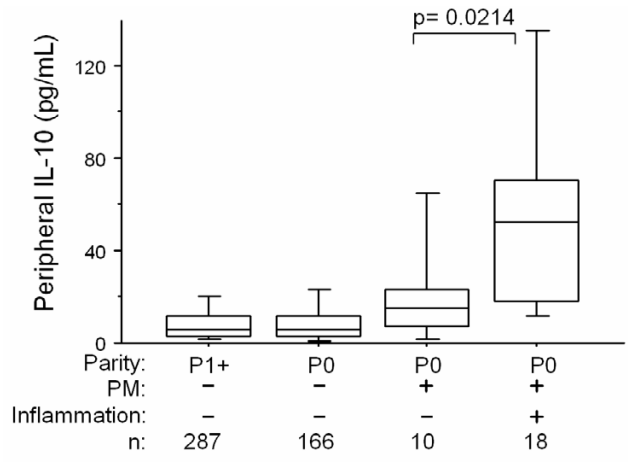

Figure 2
Peripheral IL-10 levels stratified for maternal parity, PM and the presence of inflammatory cells by placental histology. P-value was calculated using Mann-Whitney test. P0, primigravidae; P1+, multigravidae.

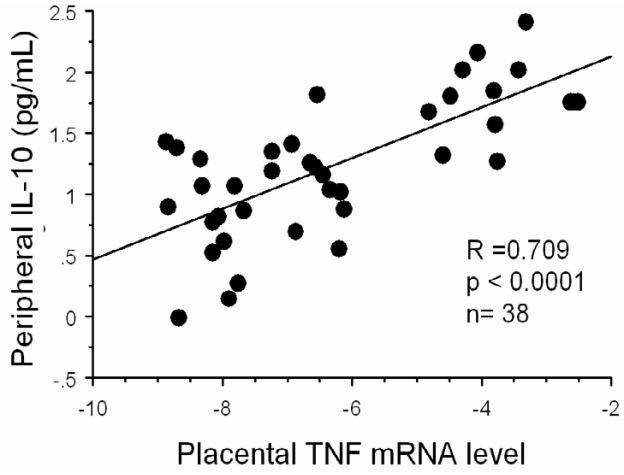

Figure 3
Relationship of peripheral IL-10 levels and placental TNF-α mRNA levels in first time mothers. Gene expression is presented as 2^{x} fold expression over KRT7. Simple regression analysis was used to calculate R and P-values.

Conclusion

In summary, these data suggest that the peripheral IL-10 level may be useful as a biomarker of inflammation due to PM. Future studies should measure antenatal levels of IL-10, and assess its relationship to parasitemia and pregnancy outcomes, and its utility for monitoring interventional trials. The sensitivity and specificity of peripheral IL-10 levels at delivery suggest that they may not be sufficient to be used clinically as diagnostic tools. Additional biomarkers of PM, placental inflammation and PM-related poor outcomes are needed to improve the clinical management of this major public health problem.

Authors' contributions

TKM, MF, and PED designed and managed the MOMS Project. ERK and JDK performed the multiplex cytokine

assay. AM performed PCR and histology studies. AM analyzed the data and wrote the manuscript with assistance from other authors.

Conflict of interest

The author(s) declare that they have no competing interests.

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Natural selection of *FLT1* alleles and their association with malaria resistance *in utero*

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Placental malaria (PM) caused by *Plasmodium falciparum* contributes significantly to infant mortality in sub-Saharan Africa and is associated with pregnancy loss. We hypothesized that fetal genes that modify PM would be associated with fetal fitness. During PM, placental trophoblasts produce soluble *fms*-like tyrosine kinase 1 (sFlt1), also known as soluble VEGF receptor 1, an angiogenesis inhibitor associated with preeclampsia. Here we present a study examining the genotype of the *fms*-related tyrosine kinase 1 (*FLT1*) 3′ UTR in Tanzanian mother–infant pairs. First-time mothers suffer the most PM, and newborn FLT1 genotype distribution differed by birth order, with newborns of first-time mothers outside of Hardy–Weinberg equilibrium (HWE) during peak PM season. Among first-time but not other mothers, maternal *FLT1* genotype was associated with a history of prior pregnancy loss. During PM, newborn *FLT1* genotype was associated with low birth weight and placental inflammatory gene expression. *FLT1* genotype was also associated with Flt1 levels among study subjects and *in vitro*. Thus, FLT1 variants confer fetal fitness *in utero* and are associated with the maternal immune response during PM. This indicates that *FLT1* is under natural selection in a malaria endemic area and that human exposure to malaria can influence the evolutionary genetics of the maternal-fetal relationship.

angiogenesis | parasitology | pregnancy | placenta | *Plasmodium falciparum*

In Africa, 30 million women living in malaria-endemic areas become pregnant each year and are at risk for placental malaria (PM), which is estimated to cause nearly one-third of perinatal mortality (1), including stillbirths (2). *Plasmodium falciparum*-infected erythrocytes adhere to chondroitin sulfate A and sequester in the maternal circulation of the placenta (3). First-time mothers have the highest rates of PM and of severe placental inflammation, which is specifically related to poor outcome. PM is less frequent and severe in multiparae, who have developed antibodies against chondroitin sulfate A-adherent infected erythrocytes (4).

Trophoblasts secrete soluble *fms*-like tyrosine kinase 1 (sFlt1) into the maternal circulation (5), and levels are elevated during preeclampsia (6, 7). Adenoviral delivery of sFlt1 in a rat model causes the features of preeclampsia: Hypertension, proteinuria, and glomerular endotheliosis (7). During PM in first-time mothers, sFlt1 levels are elevated and maternal inflammatory cells produce its ligand, VEGF, suggesting maternal-fetal conflict (8). sFlt1 has been postulated to be involved in maternal-fetal conflict over nutrient allocation (9), but sFlt1 also has anti-inflammatory effects through VEGF antagonism (10, 11), and therefore sFlt1 might modulate maternal inflammation during PM (8) and modify fetal outcomes.

A dinucleotide repeat polymorphism, rs3138582 $(TG)_{n\ 24+}$, is ≈3 kb downstream of the last exon of the *fms*-related tyrosine kinase 1 (*FLT1*) gene. Polymorphism length was not associated with renal disease in one study (12), and it has not been examined further in other human conditions. RNA structure prediction indicates that the repeat forms a stem-loop [supporting information (SI) Fig. S1], suggesting a possible role in message regulation or stability. Sequence analysis also suggests that repeat length has increased within the primate lineage. We therefore investigated *FLT1* repeat length in Tanzanian mother–infant pairs and its relationship with poor outcomes caused by PM.

Results

Dinucleotide Repeat Is Expressed Within the *FLT1* UTR. The dinucleotide repeat was included in the UTR of a *FLT1* transcript (X51602) obtained from a placental cDNA library (13); however, subsequent sequences including RefSeq NM002019.3 end 2.5 kb upstream from the repeat (Fig. 1*A*). To confirm that the repeat is expressed within the UTR of *FLT1*, we designed primers targeting the last exon and the UTR downstream of the repeat. We amplified a ≈3-kb fragment from cDNA generated from total placental RNA by using a *FLT1*-specific reverse primer (Fig. 1*B*).

***FLT1* Repeat Length Is Diverse in the Tanzanian Study Population.** The study reported here included pregnant women who enrolled in the Mother–Offspring Malaria Studies Project and delivered singleton newborns in Muheza, Tanzania, an area of intense malaria transmission (14). Of mothers having their first live delivery (nulliparous), 19% had active PM at the time of delivery, compared with 6% of mothers having their third or subsequent live delivery (multiparous). DNA for genotyping was available from 239 infants and 235 mothers from nulliparous pregnancies and 359 infants and 330 mothers from multiparous pregnancies. The repeat length distribution in Tanzania was more diverse than previously reported in countries free of malaria (Fig. 2*A*, graph). The shortest and most frequent (24 repeats) polymorphism had a frequency of 36% compared with reported frequencies within the United States and the United Kingdom of 68% (13) and 88% (12), respectively.

The frequencies of dinucleotide repeat polymorphisms >27 repeats in length formed a separate normal distribution, and these were classified as the long (L) allele of the repeat polymorphism, with those 27 repeats or less classified as the short (S) allele. SNPs that flanked the dinucleotide repeat region and that varied in frequency between Caucasian and Yoruba populations were identified by using the International HapMap (15). These SNPs were sequenced for individuals in the study who were homozygous for discrete dinucleotide repeat lengths. The SNPs were linked to the S and L alleles (Fig. 2*A*, boxes below graph),

Author contributions: A.M., M.F., T.K.M., and P.E.D. designed research; A.M., M.F., J.L., T.K.M., and P.E.D. performed research; A.M. and P.E.D. analyzed data; and A.M. and P.E.D. wrote the paper.

The authors declare no conflict of interest.

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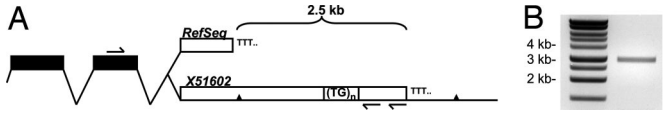

Fig. 1. The dinucleotide repeat is expressed within the *FLT1* UTR. (*A*) Diagram of the *FLT1* 3′ UTR, not drawn to scale. Coding exons are indicated in black and the UTR in white, showing both the RefSeq annotation and X51602, which includes the dinucleotide repeat. The locations of the two SNPs examined in this study are indicated as triangles. Primer sites for both the reverse transcriptase reaction and PCR are indicated. (*B*) Amplification of a ≈3-kb product using primers encompassing the last exon of the FLT1 gene and the dinucleotide repeat (expected size, 3436 bp).

indicating that the S and L alleles form discrete haplotypes. SNPs associated with Caucasian ethnicity were linked to S alleles and those associated with African ancestry to L alleles. The frequency of the S allele did not differ by maternal affiliation with the major tribes of Bondei and Sambaa or the >50 minor tribes ($n = 457$, χ^2 $P = 0.40$) living in our study area under endemic malaria transmission. The genotypes of all subjects were within Hardy–Weinberg equilibrium (HWE) ($n = 1159$, $P = 0.99$).

Infant *FLT1* Genotype Differs by Parity and Birth Season. PM is most frequent and most severe in first-time mothers; therefore infant and maternal genotypes were stratified by maternal parity (Fig. 2*B*). Overall, the frequency of the S allele was similar between infants and mothers. However, genotype distribution among infants, but not mothers, differed by parity. Fewer S-homozygous (SS) offspring were born to first-time mothers than to multiparous mothers. Although not significant, there was a trend toward increased numbers of SS offspring born to multiparous mothers. Allele frequencies are reported in Table S1. PM had a seasonal variation during this study that peaked during May–September (with PM in 28% of first-time mothers) and troughed during October–April (with PM in 15% of first-time mothers). During the peak PM season, significantly fewer SS infants were born to first-time mothers than to multiparous mothers (Fig. 2*C*). Infants born to first-time mothers during peak PM season were outside of HWE ($P = 0.018$), consistent with disproportionate mortality for SS fetuses. All other groups were within HWE.

***FLT1* Genotype Is Associated with History of Pregnancy Loss and Low Birth Weight.** The proportion of mothers who reported prior pregnancy loss varied by parity and FLT1 genotype (Fig. 3*A*). Among mothers delivering their first live infant in our study, SS mothers reported the highest rate of a prior pregnancy loss, whereas none of 46 L-homozygous (LL) mothers reported a pregnancy loss, and heterozygous (SL) mothers had an intermediate rate of loss. Thus, rates of prior pregnancy loss among

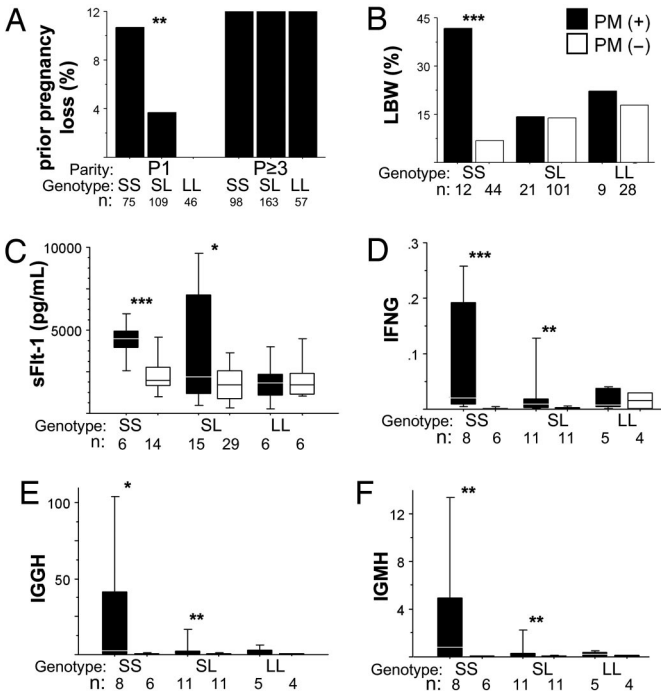

Fig. 3. Association of *FLT1* genotype with pregnancy outcome. (*A*) Rates of reported prior pregnancy loss of women stratified by parity and maternal genotype. P1, nulliparous pregnancy; P≥3, multiparous pregnancy. (*B*) Rates of LBW delivery for first-time mothers stratified by newborn genotype and PM status. (*C*) Maternal peripheral plasma sFlt1 levels of first-time mothers stratified by newborn genotype and PM status. (*D–F*) Placental transcript levels of inflammatory genes in first-time mothers stratified by newborn genotype and PM status. Transcript levels are shown as fold change relative to cytokeratin 7. *P* values calculated by χ^2 test for categoric variables and *t* test for log-transformed sFlt1 levels and corrected cycle threshold (CT) values. *, $P < 0.08$; *, $P < 0.05$; ***, $P < 0.01$.

nulliparae were consistent with the likelihood of having an SS fetus. Reports of multiparous mothers reflected a cumulative increase in prior pregnancy losses, with all genotypes reporting a similar rate of loss. The data suggest that mothers experience excess loss of LL offspring after their first pregnancy; however, data regarding pregnancy order of the prior losses were not available.

Low birth weight (LBW) is a complication of PM frequently observed in first-time mothers and predicts increased infant mortality. Rates of LBW were elevated in SS offspring, but not SL or LL offspring, born to first-time mothers with PM (Fig. 3*B*). In contrast, SS offspring of PM-negative first-time mothers had

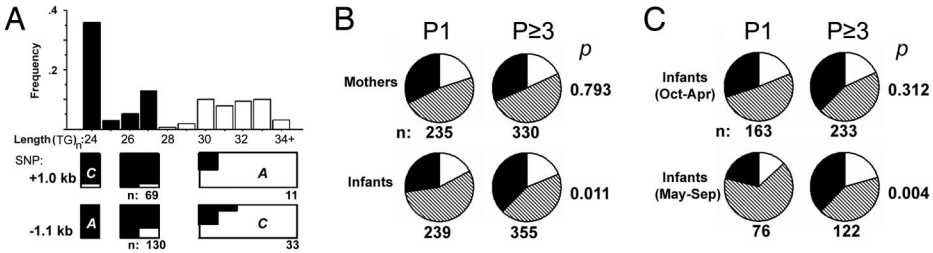

Fig. 2. Newborn genotype at the *FLT1* locus differs according to maternal parity and season of birth. (*A*) Frequency distribution of *FLT1* 3′ UTR repeat lengths, with S and L allele classification shown on graph as black and white bars, respectively. Relative proportion of flanking SNP variants, for individuals homozygous for specific repeat lengths with the reference allele (i.e., allele most common in Caucasians), is shown as black in boxes below graph. (*B*) Genotypes of mothers and their newborns, shown as proportions stratified by parity. (*C*) Genotypes of newborns according to season of birth, shown as proportions stratified by maternal parity. The proportions of SS and LL homozygotes are represented in black and white, respectively, with the SL heterozygotes in a hatched pattern. *P* values were calculated by using χ^2 test (2 × 3) across all genotypes. P1, nulliparous pregnancy; P≥3, multiparous pregnancy.

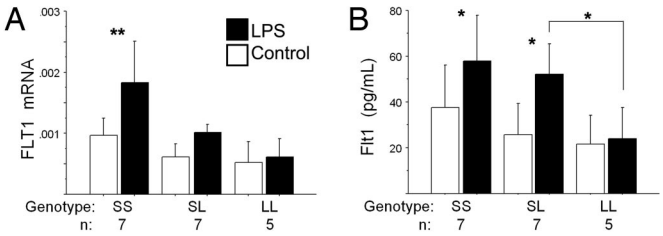

Fig. 4. Association of *FLT1* genotype with expression levels *in vitro*. (*A*) Transcript levels of the transmembrane isoform of FLT1 and (*B*) protein levels of Flt1 in supernatants of cultured PBMC stratified by genotype and LPS treatment (100 ng/ml for 24 h). *P* values calculated by paired *t* test for log-transformed Flt1 levels and corrected CT values. *, $P < 0.05$; **, $P < 0.01$.

the lowest rates of LBW among all groups of children. Maternal genotype was not associated with LBW during PM. Rates of PM did not differ by maternal or infant genotype (data not shown), consistent with the role of maternal acquired immunity as the major determinant of PM risk. Rates of hypertension were not associated with maternal or infant *FLT1* genotype.

Infant *FLT1* Genotype Is Associated with Placental Inflammation. Inflammatory responses are associated with pregnancy loss, and animal studies indicate that immune-mediated pregnancy failure is a consequence of immune activation at the maternal-fetal interface (reviewed in ref. 16). We earlier observed that during PM, sFlt1 was specifically elevated in mothers who had placental inflammation by histology (8). sFlt1 levels in maternal plasma were elevated during PM in first-time mothers of SS offspring, but not LL offspring (Fig. 3*C*), and were heterogenous in mothers of SL offspring. Placental transcript levels of IFN-γ and IgG and −M heavy chains were previously associated with poor pregnancy outcome in this cohort (17) and were elevated in the placentas of SS and SL but not LL infants during PM (Fig. 3*D–F*). The placentas of SS infants had the greatest levels of these transcripts. sFLT1 and IFN-γ placental transcript levels approached significant correlation ($R = 0.392$, $n = 24$, $P = 0.0584$).

***FLT1* Genotype Is Associated with Expression Levels *In Vitro*.** To determine whether dinucleotide repeat length may be a functional polymorphism, we examined the effect of LPS on FLT1 expression in peripheral blood mononuclear cells (PBMC) obtained from cord blood from infants of different genotypes. LPS stimulation of PBMC from SS and SL infants resulted in increased *FLT1* mRNA and protein expression, whereas PBMC from LL individuals did not increase FLT1 expression (Fig. 4). PBMC from SS and SL individuals also had higher levels of FLT1 expression compared with LL individuals.

Discussion

PM is a major public health problem in tropical countries, and first-time mothers are particularly affected by poor outcomes. Our data suggest that *FLT1* genotype is causally related to pregnancy outcome during PM. This may be a direct effect of 3′ UTR dinucleotide repeat length, or alternatively, length may be linked to a distinct causative site. We observed alleles of 24–34 repeats in this study. Data indicate that the chimpanzee allele contains 20 repeats, whereas the rhesus and mouse contain 10–12 repeats, suggesting recent expansion in the human lineage. The dinucleotide repeat is predicted to form a stem-loop, thus repeat length may alter RNA secondary structure (Fig. S1) and regulate mRNA stability, splicing, or translation. Subsequent mRNA processing may affect the regulation of Flt1 production or downstream Flt1 signaling in the fetoplacental unit.

Functional data using PBMC suggest that the polymorphism has an effect on *FLT1* mRNA and protein expression in response to LPS stimulation. A major caveat is that during pregnancy and during PM, the trophoblast is the major source of the soluble isoform of FLT1, and the trophoblast possesses unique *FLT1* regulatory mechanisms (18). The effect of this polymorphism on trophoblast *FLT1* expression remains to be determined.

Poor outcomes during PM are associated with placental inflammation, and the infiltrating immune cells are of maternal origin. We observed that infant *FLT1* genotype was associated with pregnancy outcome during PM, and therefore we postulate that infant *FLT1* genotype may modulate the maternal inflammatory response to PM. Two observations support this model: First, infant *FLT1* genotype was associated with inflammatory gene expression in this study, and second, placental sFlt1 levels were previously associated with inflammation during PM (8). These relationships are intriguing because sFlt1 has anti-inflammatory effects in experimental models (10, 11). Further evidence of causation between *FLT1* genotype, maternal inflammation, and sFlt1 expression during PM will require longitudinal data during PM episodes or an experimental model of PM.

Our data from Tanzania suggest that maternal malaria exerts selective pressure *in utero* at the *FLT1* locus through pregnancy loss. SS offspring seem to be at a selective disadvantage in first-time mothers in malaria endemic areas. The data may also suggest that SS offspring are at an advantage in the absence of malaria pressure: SS offspring were slightly enriched among malaria-immune multiparous mothers, and LBW was least frequent among SS offspring born to PM-negative mothers, although neither trend attained significance. The effect of this polymorphism on pregnancy outcomes in nonmalarious areas has not been characterized, although populations in the United States and United Kingdom have a large preponderance of the SS genotype. The natural role of sFlt1 in pregnancy is not known; however, it may contribute to the pathogenesis of preeclampsia. Preeclampsia, like PM, is most frequent in first-time mothers, and sFlt1 levels are elevated in healthy first vs. second pregnancies (19). We speculate that exposure to malaria during human evolution influenced sFlt1 regulatory mechanisms, and this might continue to affect pregnancies outside of malaria endemic areas.

This study reports a human gene that confers resistance to infectious disease *in utero*. Because this was a community-based study with a high rate of disease, we were able to observe natural selection occurring in a human population. During a major malaria epidemic in Sri Lanka during 1934–1935, PM caused perinatal death in 67% of cases with half occurring *in utero* (20). Therefore epidemic PM, which largely affects nonimmune populations, may lead to large-scale selective sweeps, whereas endemic PM may influence the genotype of only first-born offspring whose mothers lack immunity to chondroitin sulfate A-adherent infected erythrocytes. These data suggest that fetal genes that modify maternal inflammation may be under natural selection secondary to malaria, thereby contributing to the evolution of the human maternal-fetal relationship.

Methods

Human Subjects. The study reported here included nulliparae and multiparae who enrolled in the Mother–Offspring Malaria Studies Project, delivering at the Muheza Designated District Hospital between September 2002 and April 2005. Cord blood for PBMC isolation was collected from deliveries occurring at the Morogoro Regional Hospital during 2007. Women provided informed consent for themselves and their offspring. Birth weight was measured on digital scales and LBW defined as ≤2,500 g. PM was diagnosed by microscopic examination of placental blood obtained by mechanical extraction from the placenta after delivery.

***FLT* UTR Amplification.** cDNA was generated from placental RNA and isolated as described in a following section by using SuperScript III (Invitrogen) and a

FLT1-specific reverse primer TGCCACAGGATGTTTTAACG. PCR was performed by using ex-Taq polymerase (TaKaRa) and the primers CTTCACCTGGACTGACAGCA (forward) and GGTTCGAAAACCCCATACAA (reverse) with an annealing temperature of 59°C for an expected product size of 3,436 bp.

***FLT1* Genotyping.** DNA was extracted from filter–paper blood spots and frozen blood pellets (Qiagen). The *FLT1* 3′ UTR dinucleotide repeat rs3138582 was PCR amplified by using AmpliTaq gold (ABI) and the primers TGGCCGACAGTGGTGTAAC (forward) and AACTTTAAAATTCCAGTTTCCTTAAA (reverse) with 5′ 6-FAM modification of the forward primer and an annealing temperature of 50°C. Fragment length was determined by capillary electrophoresis (ABI). The following SNPs were PCR amplified: rs9554314 (C/A) at −1.14 kb and rs17086497 (A/C) at +0.99 kb from the dinucleotide repeat. The following primer pairs were used: AGCAATCCACTGTTGCCTCT (forward) with GGGAGACAGGGTAGGAAAGG (reverse) and TTTCCAGAGCCATGAGAACA (forward) with GGCAAGAGGCATTTTGTCTT (reverse) for each SNP, respectively. PCR products were purified and sequenced by using the reverse primers for each SNP.

Flt1 ELISA. sFlt1 levels in maternal plasma were assayed as previously described (8). Briefly, peripheral blood was collected in citrate phosphate dextrose immediately after delivery, and plasma was separated and frozen at −80°C. Soluble Flt1 levels in peripheral plasma were determined in duplicate by ELISA kit DVR100A (R&D Systems). Cell culture media was centrifuged and Flt1 levels determined in duplicate by ELISA kit DVR100B (R&D Systems).

Quantitative PCR. Placentas were collected at delivery, and fresh tissue was frozen in liquid nitrogen. Total RNA was extracted from cryosections as previously described (17) or harvested from PBMC samples by using RNeasy Mini Kits (Qiagen). cDNA was synthesized by using a SuperScript III enzyme (Invitrogen Life Technologies) and anchored oligo$(dT)_{20}$ primers. Real-time PCR was performed in duplicate by using SYBR Green Master Mix and an ABI Prism 7500 system (Applied Biosystems). Quantitative PCR was performed by using previously specified primers (17) in addition to the transmembrane isoform of FLT1: AGGGGAAGAAATCCTCCAGA (forward) and GAGGTTTCGCAGGAGGTATG (reverse) and GAPDH: ACTTCAACAGCGACACCCACTC (forward) and CACCCTGTTGCTGTAGCCAAA (reverse). Threshold cycles (CT) were calculated and normalized to the CT of KRT7 or GAPDH for placental or PBMC samples, respectively.

Cell Culture. Cord blood samples were obtained immediately after delivery. PBMC were isolated by using lymphocyte separation media, frozen in RPMI with 40% FCS and 10% DMSO, stored in liquid nitrogen, and shipped to Seattle on dry ice. *FLT1* genotype was determined as previously described. Cell viability was determined by trypan blue exclusion, and cells were plated at 1×10^6/ml. Cells were cultured in RPMI-1640 (Sigma) supplemented with 10% heat-inactivated FCS, penicillin, streptomycin, and gentamicin. After 24 h of incubation, 100 ng/ml LPS from *Escherichia coli* O55:B5 (Sigma) was added for another 24 h.

Statistical Analysis. Analyses were performed by using Statview (SAS). *P* values were calculated by χ^2 test for categorical variables, and *t* test for log-transformed Flt1 levels and corrected CT values. For cell culture assays, paired *t* test was used. Regression coefficients were calculated by using simple regression analysis. A *P* value of <0.05 was considered significant.

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